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# INVESTIGATION OF CRIMEAN-CONGO HEMORRHAGIC FEVER AND HEMORRHAGIC FEVER WITH RENAL SYNDROME IN GREECE

Annual Report

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## TABLE OF CONTENTS

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From April 1987 through April 1988, 597 blood samples were collected from residents of 4 counties of northern Greece. Anti-bodies were detected in 8 and 7 individuals against Hantaan virus and Crimean-Congo Hemorrhagic Fever respectively. Two hundred and two blood samples were taken from patients with disease resembling HFRS and one hundred and sixty nine from patients with disease or syndromes resembling CCHF. Serological diagnosis showed that 7 patients were infected by Hantaan virus, whereas there was no evidence for recent infection by CCHF virus. Two out of 54 small rodents trapped were found to have anti-bodies against Hantaan virus. 29 pools of ticks were collected for CCHF virus isolation. Virus isolation from rodents (Hantaan) and ticks (CCHF) are in progress.

Finally, the research performed for HFRS and CCHF viruses in Greece until April 1988 is discussed ~~below~~ and the conclusion drawn are herein reported. Keywords: Epidemiology. (AW) K

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# FOREWORD

For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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## A. INTRODUCTION

During a 12-month period, from April 1987 through April 1988, human serosurveys for Crimean-Congo hemorrhagic fever (CCHF) and Hemorrhagic Fever with Renal Syndrome (HFRS) were conducted in several counties of Greece. New human cases of HFRS were diagnosed, the ELISA IgM capture method was applied for the early diagnosis of the disease and small mammals were trapped for Hantaan virus isolation. The results of the ELISA IgM capture technique were compared with those of the IFA test.

The study on Congo-Crimean hemorrhagic fever virus, included identification of new endemic foci, collection of ticks for virus isolation, and attempts to diagnose the disease.

Finally, the research performed on HFRS and CCHF in Greece up until April 1987, was discussed and the conclusions drawn are reported.

## B. HEMORRHAGIC FEVER WITH RENAL SYNDROME (HFRS)

### B1. Human Serosusrvey

During the period, April 1987 through April 1988, 579 blood samples were collected from residents of four counties of northern Greece (Evros, Rodopi, Halkidiki, Ioannina) (Fig. 1). In addition 81 blood samples were collected from soldiers serving in northern Greece. Rodopi and Evros counties were selected for the serosurvey because of their proximity to the Greek-Bulgarian borders, since the Bulgarian Rodopi area is a highly endemic area for HFRS as reported by Bulgarian scientists. Previous serosurveys and attempts to diagnose HFRS cases, showed that the Greek Rodopi area is not an HFRS endemic area.

All the blood samples were identified according to age, sex, occupation, previous travel history (mainly abroad) and residence of the donors).

Analysis of the epidemiological data on the sex distribution are shown in Table 1. Out of 580 examined individuals, 388 were males and 192 females. The occupational distribution is shown in Figure 2. Of 580 individuals, 406 were farmers, 58 wood-cutters, 11 shepherds, 24 workers in a Public Health Veterinarian Station and 81 army soldiers serving in northern Greece. The sex and age distribution of the samples examined during the 12-month period, is very similar to the distribution of 3,388 individuals examined for specific antibodies to Hantaan and CCHF viruses from 1982 to 1987 (figures 3,4,5,6,7,8).

All human sera collected from April 1987 to April 1988, were kept at -20°C until tested for anti-Hantaan virus antibody by IFA test with goat anti-human fluorescence immunoglobulin. The spot-slides contained Vero E-6 cells, approximately 50% of them in-

fected with the 76-118 strain of Hantaan vitus prototype. The sera were considered as positive if characteristic cytoplasmic fluorescence was detected at the 1:16 dilution.

In previous serosurveys conducted in Greece, between 1982 and 1987 a total number of 3,388 human sera were examined by IFA test. The overall antibody prevelance rate was 1.9% with a range from 0 to 14%. The highest percentage of seropositivity occured in areas where clinical cases of the disease were diagnosed. Moreover, seropositives were detected in 22 out of 24 countries (in a total of 54 Greek counties) where serosurveys were conducted (Figure 9).

Analysis of the data showed that the males are more frequently infected than females, and that the most common groups infected are between 31 to 50 years (Figure 10).

The results of the current study which was conducted in four counties are shown in Figures 11, 12, 13 and 14. Six seropositives were found in total, living in 3 out of 4 counties (Figure 1). The highest prevelance of seropositivity (2.7%) occured in Ioannina county where most of the HFRS clinical cases were diagnosed. For the first time, seropositives were found in Evros and Rodopi counties which are neighbouring on Bulgaria. Male individuals are more frequently infected than females and the high risk occupations are wood-cutters, farmers and shepherds (Figure 14).

**B2. Disease**

Acute-phase and convalescent-phase sera were collected during the period from April 1987 to April 1988, from 195 patients whose illness was diagnosed as either HFRS, or leptospirosis, or pyrexia of unknown origin and from 5 patients with pyrexia and hemorrhagic manifestations (Table 2). The patients were residents

of various parts of Greece and were hospitalized in local hospitals or refered to the University Clinics of Thessaloniki and Ioannina for specialized diagnosis and treatment.

Samples of the patient's serum, single or paired, were examined on the day of arrival in the laboratory or kept at -20°C until tested by the indirect immunofluorescence antibody (IFA) test for both IgG and IgM specific to Hantaan virus antibodies. All sera taken from patients suspected of having HFRS were also examined by the enzyme-linked immunosorbent assay (ELISA IgM capture) for the detection of specific IgM antibodies to Hantaan virus. Sera were tested at two-fold dilutions (initial dilution 1:16) by IFA with fluorescein-labelled goat anti-human immunoglobulin on spot-slides containing Vero E-6 cells. Approximately 50% of the cells were infected with the 76-118 strain of prototype Hantaan virus. In the ELISA IgM capture technique, sera were tested at two-fold dilutions (initial dilution 1:100). The method of the ELISA IgM capture is described elsewhere (page). The medical records of patients with serologically confirmed HFRS were also reviewed.

The diagnosis of HFRS was serologically confirmed in seven of the examined patients whose clinical findings were in accordance with the symptomatology of the disease (Table 2). Six of the patients were residents of Ioannina county and 1 of Drama county (Fig. 15). The results of the serological diagnosis by both the IFA and the ELISA IgM capture tests are shown in Table 3. In five of those patients the disease was severe with abrupt onset, fever, flushing over the face and the neck, conjuctival injection, hemorrhagic manifestations and acute renal insufficiency. Six of the patients susrvived but one died during the oliguric phase of the disease. He developed severe hemorrhagic manifestations, nasal bleeding, hematuria and gross gastrointestinal bleeding.

Up todate 41 HFRS cases have been serologically diagnosed in Greece. The clinical features of the disease in Greece seem to be quite similar to those of the severe form of HFRS in Asia; that is abrupt onset, fever, flushing over the face and neck, conjuctival injection and a high incidence of severe systemic manifestations requiring aggressive treatment. According to the medical records of 27 Greek patients with HFRS, sixteen had clinically significant hypotension, with progression to shock in 8 patients, 12 became precomatose or comatose and 10 had renal failure with oliguria or anuria, requiring hemodialysis.

The indication for dialysis in these patients was serum urea levels > 250 ng/dl, serum creatinine levels > 8 mg/dl, serum bicarbonate levels < 10mEq/L, pH < 7.2 and serum potassium > 7 mEq/L, associated with oliguria or anuria and not responding to diuretics and fluid restriction. However the most important criterion was the level of serum K+. Six patients developed severe hemorrhage, three had platelet counts < 50,000 cells/ml at the time of hemorrhage. The clinical and laboratory findings are shown in Tables 4 and 5 and Figures 16 and 17.

Hemorrhagic fever with renal syndrome in Greece appears in late March and lasts until early November with a peak in August (Figure 18). The disease is widespread in Greece and may appear as isolated cases or outbreaks, particularly when groups such as wood-cutters and farmers have to spend the night outdoors or in temporary quarters thereby being exposed to infected rodents.

So far 41 cases of HFRS have been serologically diagnosed in Greece with 7 deaths reported (mortality rate 17%). The disease has been diagnosed in residents of 10 counties in Greece (Table 6, Figure 19). As is shown in Table 6 and Figure 19, the majority of the cases (27 out of 41) occured in Ioannina county which is close to the Albanian border.

Males are more frequently infected than females (Figure 20) and the high risk occupational groups are the wood-cutters, farmers and shepherds (Figure 21).

According to our findings, 2 out of 10 counties where the disease was serologically diagnosed can be characterized as high-risk endemic areas (Figure 20). In Ioannina county, 27 HFRS cases have been diagnosed and six patients died. Also in Pella county which is close to the Yugoslavian border 5 HFRS cases have been diagnosed with no deaths.

As for the counties adjacent to the Bulgarian border (Serres, Drama, Xanthi, Rodopi, Evros) only two HFRS cases have been diagnosed, one in Serres and one in Drama county. Previous reports from Bulgaria as well as information given from Bulgarian scientists suggest that the Bulgarian Rodopi area is a highly endemic HFRS area as opposed to the Grecian Rodopi area. The serosurveys, the geographic distribution of the disease and the small mammal surveys, demonstrated that only the western part of this area (Serres and Drama counties) can be characterized as endemic. Thanks to the excellent collaboration of the County Hospital located in the Greek Rodopi area for the past three years, blood samples collected from patients suspected of having HFRS were sent to our laboratory for serological diagnosis. The Greek Ministry of Health and Welfare had taken care of sending information booklets (written by us), with the description of the clinical picture of the disease and means of blood sample transportation.

Despite these efforts only two HFRS cases have been diagnosed in this area. The results from the serosurveys we conducted in these areas revealed that the Greek Rodopi area is not a high-risk HFRS endemic area when compared to the findings from serosurveys conducted elsewhere in Greece.

## B3.1. Attempts at Hantaan virus isolation from captured rodents

Our aim was to trap small rodents from areas where HFRS cases were serologically diagnosed in past and recent years, in order to isolate Hantaviruses. The localities where the small rodents were trapped are shown in Figure 22.

The IFA test showed two rodents with antibodies to Hantaan virus (Table 7). The spleen, lungs and kidney samples of the positive rodents as well as those of two negative rodents, were inoculated in Vero E-6 cells for virus isolation.

Fifteen days later, spot-slides were prepared from the Vero E-6 cells and the IFA test was performed using mouse positive serum for the detection of the Hantaan virus. Cells harvested from one flask which was inoculated by lung emulsion (received from a seropositive Ap. flavicollis mouse) showed approximately a 4% fluorescence. After the second pass of the infected cells (cocultivation with normal uninfected cells) -fifteen days later- when tested again by IFA test for Hantaan virus infection the cells were found negative for Hantaan virus. The supernatant of the first pass as well as the supernatant of the initial flask were kept in -70°C.

We believe that a Hantaan virus exists in those supernatants but we cannot recover the virus. Yugoslavian scientists had the same problem (Personal communication with Dr. Ana Gligic).

In previous studies for the identification of the host of the Hantaan virus which exists in Greece (Table 8) as well as in our recent study, antibodies were detected in Apodemus flavicollis trapped in wooded HFRS endemic areas >1,200 m above sea level.

Two house rats (Rattus rattus) captured in a slaughter house in Thessaloniki were found to be seropositive (Table 2).

Todate, neither Apodemus agrarius, the host of Hantaan virus, nor Clethrionomys glareolus, the host of Puumala virus, have been captured at any site sampled.

**B3.2. Attempts to isolate Hantaviruses from HFRS patients**

In March 1986 a Hantaan virus was isolated from the urine of a severely ill HFRS patient. Both whole blood and urine were obtained on the seventh day of ilness, when the patient improved slightly after the initial dialysis treatment. Serological comparison of the isolated virus to other Hantaviruses establishes is as a member of this group. Although the IFA tests results revealed little difference between the isolate and Hantaan virus, the more specific PRN tests demonstrated sufficient differences to suggest that the isolated virus presents a unique strain of Hantaviruses. A comparison of PRN tests to both Hantaan virus and the Greek isolate with titers of convalescent sera from patients previously diagnosed for severe HFRS, demonstrated higher titers to the Greek virus (Table 9), a result suggesting that this virus may have been responsible for many of these cases.

During the period from April 1987 through April 1988, blood, serum and urine samples were taken from 3 HFRS patients on the 5th, 6th and 7th day of their disease. The patients were hospitalized in Ioannina General Hospital and all samples were sent by air to our laboratory within 4 h.

Upon arrival, 1-ml aliquots of whole blood and serum were inoculated onto Vero E-6 cells grown in 25-ml plastic flasks. Aliquots of 0.25 ml of urine (immediately alkalized with sodium bicarbonate) were also inoculated into 25-ml flasks of Vero E-6 cells. Inoculated flasks were incubated for 15 days at 37°C, then the

cells were suspended, most were transfered to fresh flasks, and 10-well spot-slides were prepared for the rest and examined for characteristic hantavirus cytoplasmic fluorescence, using reference antibodies to Hantaan virus. After a further 15 days of inoculation all cells which were taken from the corresponding flasks with the sample inoculum were negative. On subsequent passage the cells were again negative and further attempts were stopped.

**B4. Application of the Enzyme-Linked Immunosorbent Assay (ELISA) for the Diagnosis of Hemorrhagic Fever with Renal Syndrome (HFRS)**

The ELISA IgM-capture method was applied to the diagnosis of the Hemmorrhagic Fever with renal syndrome (HFRS). The sensitivity as well as the specificity of this test was compared to the IFA test which is currently used for the diagnosis of the disease.

Fifty-six serum samples were taken from 19 HFRS patients at intervals from the onset of the disease up to one year.

Also 131 serum samples were taken from patients with rheumatoid arthritis (20 samples), with macroglobulinemia (3 samples), with pyrexia of unknown origin (41 samples) and from healthy individuals living in HFRS endemic areas (67 samples). All the above sera were tested by both ELISA IgM capture and IFA test for the detection of specific IgM antibodies to Hantaan virus.

The patients' sera were diluted in SerDil which contained fetal bovine serum and Tween-20 to reduce non-specific binding. HTN antigen and control (negative) antigen were added to the SerDil to allow specific captured IgM viral antibodies to bind to the antigen.

Anti-HTN antibody and anti-species antibodies conjugated to HRPO were also diluted in SerDil. The patients' total IgM, sequestered on the solid phase, were tested for virus specificity by incuba-

tion with Hantaan virus antigen, strain 76-118 (whole virus concentrated and inactivated). The diluted patients' sera were incubated in a microtiter plate (coated overnight at 4°C with anti-human IgM antibody in PBS pH 7.4) for 60 min at 37°C. After careful washing (thrice), antigen diluted to 1:40 in SerDil was added to the plates and a 60 min incubation at 37°C followed. After washing, rabbit IgG antibodies to Hantaan virus were added, followed by enzyme peroxidase-labelled anti-rabbit IgG antibody for 60 min incubation periods respectively. The test was read in a Flow photometer at 405 nm filter.

Immunofluorescent-antibody assays used Vero E-6 cells that had been infected with Hantaan prototype virus, fixed to 10-well spot slides and stored at 20°C before use. Anti-human IgM conjugated was also used.

Comparison of titers obtained by IFA assay and ELISA IgM capture assay (EIA) respectively, on the first serum sample submitted for serological confirmation of HFRS, showed that the latter are much higher, confirming the increased sensitivity of the method (Table 10).

According to our results, the sensitivity of the IgM ELISA capture was approximately 200 times greater than that of the IFA method when testing blood serum samples within the first 10 days of illness, 250 times greater when testing serum samples from patients in the 11th to 20th days of their illness, and a 100 times greater in blood samples from patients in the 21st to 30th days of the illness (Figure). These differences are significant for $P < 0.0005$, $P < 0.005$ and $P < 0.02$ respectively. For thirty one to sixty days time span the difference in the sensitivity of the two methods is relatively unimportant $0.1 < P\ 0.2$ for the specific degrees of freedom (D=5). (Figures 23, 24).

Studying the specificity of the tests, all 131 serum samples taken from non-HFRS individuals were found negative by both ELISA and IFA tests. In conclusion, according to our results the ELISA IgM capture immunoassay is more sensitive than the IFA assay but further attempts need to be made to determine the degree of specificity of the EIA test. Since all acute and convalescent sera were positive by IgM ELISA this test could become an important tool for early diagnosis in acute human HFRS infections.

## C. CRIMEAN CONGO HEMORRHAGIC FEVER (CCHF)

### C1. Human serosurvey

During the period April 1987 through April 1988, 579 blood samples were collected. These blood samples were the same ones that we used for the HFRS serosurvey. In addition, 81 blood samples were collected from the residents of Anilio village (Ioannina county) because of a CCHF seropositive individual who was identified in that area in 1986.

We examined all the sera we obtained by IFA and ELISA tests. The antigen fot the IFA test was prepared by dropping (50% vero E-6 cells infected with CCHF virus strain IbAn10200 which was obtained from the Yale Arbovirus Research Unit (Dr. R. Shope) on a 12-circle printed slide and fixing with acetone. The sera were considered as positive if characteristic cytofluorescence was detected at the dilution 1:4. For the ELISA (IgG capture) assay, the examined sera were diluted in Serum Diluent (SerDil) with fetal bovine serum (FBS) and Tween-20 to reduce non-specific binding. CCHF antigen and control (negative) antigen were added to the serum to allow specific capture IgG viral antibodies to the antigen. Anti-CCHF antibody and antispecies antibodies were also diluted in serum diluent. The examined total IgG of the serum, sequestered on the solid phase, was tested for virus specificity by incubation with CCHF virus antigen, strain IbAn10200 (supernatant from Vero E-6 cells infected by the CCHF virus, unconcentrated and inactivated by beta-propiolactone). The diluted sera were incubated in a microtiter plate (coated overnight at 4°C with anti-human IgG antibody in PBS pH 7.4) for 60 min. at 37°C. After careful washing (three times), antigen diluted to 1:40 in serum diluent, was added to the plates and a

60 min. incubation at 37°C followed. After washing, mouse IgG antibodies to CCHF virus were added, followed by enzyme peroxidase-labelled anti-mouse IgG antibody for 60 min. incubation periods each time. The test was read at 405 nm filter. The sera were considered as positive at a dilution 1:100.

Analysis of the epidemiological data on sex and age distribution are shown in Table 1. Out of 579 examined individuals, 387 were males and 192 females. The occupation distribution is shown in Figure 2. Of the 579 individuals, 405 were farmers, 58 wood cutters, 11 shepherds, 25 workers at a Public Health Veterinarian Station and 81 army soldiers serving in northern Greece. The sex and age distribution of the samples examined during the 12-month period, is very similar to the distribution of 3,388 individuals examined for specific antibodies to Hantaan and CCHF viruses from 1982 to 1987 (Figures 3,4,5,6,7,8). In previous serosurveys conducted in Greece a total number of 3,388 human sera were examined by the IFA test. The overall antibody prevelance rate was 1% with a range from 0 to 9.9%. The highest percentage of seropositivity was found in Imathia county (site of CCHF virus isolation) and in Ioannina county (Anilio village). Seropositives were detected in 12 out of 24 counties where serosurveys were conducted.

Analysis of the data showed that males are more frequently infected than females and that the most common groups infected are between 41 to 60 years (Figure 26) while the high risk occupational groups include farmers, shepherds and wood-cutters (Figure 27).

The results of the current study which was conducted in four counties are shown in Figure 28. In total, 7 seropositives living in 3 out of 4 counties were identified. Six of the 7 seropositives were farmers and five were residents of Anilio village in Ioannina county (Table 11).

As previously mentioned the highest prevelance of seropositives (Figure 25) occured in Ioannina and in Imathia counties (the site of virus isolation).

Recent information from the Bulgarian scientist Dr. Vasilenko during the 1st International Symposium on Hantaviruses and Crimean Congo Hemorrhagic Fever virus, in Porto Carras, Halkidiki, Greece (September 1988) indicate that eastern Bulgarian Rodopi is a high-risk CCHF area (Figure 29) whereas for the Greek Rodopi area the recent investigation shows that little evidence exists to characterize this area as endemic. In Greece, attention must be paid to the counties of Imathia and Ioannina, which is close to Albania where recently CCHF human cases have been reported.

C2. HFRS Disease

Blood samples were collected from patients in General Fospitals of endemic areas (Ioannina, Veria, Halkidiki) as well as from hospitals close to the Bulgarian border (Rodopi, Evros). We examined by IFA and ELISA IgM capture methods 169 blood samples (76 single, 93 paired) taken from patients with pyrexia of unknown origin (111), patients with disease resembling CCHF (16) and patients with pyrexia of unknown origin as well as elevated liver enzymes (SGOT-SGPT) (Table 12). None of the patients was found to be infected by the CCHF virus.

## C3. Attempts for virus isolation from ticks

### a. Tick collection

Ticks were collected from the body of animals. Two counties were close to the Bulgarian border (Serres and Rodopi), two counties were found of having a high percentage of seropositives in humans (Ioannina, Imathia) and finally two counties were found to have seropositives (Kilkis, Halkidiki).

The identification of the ticks was made by the veterinarians who work at the Animal Infectious Diseases Department of the Veterinary School at the Aristotelian University of Thessaloniki.

Upon identification, pools were assembled to contain 10-15 ticks of the same species (Table 13) labelled with the date and place of collection. The pools were stored at -70°C until used.

### b. Virus isolation technique

Pooled ticks were ground in a mortar and PBS buffer pH 7.2 enriched with 1% bovine serum albumin (fraction V) and penicillin/streptomycin was added to make up a 10% suspension approximately. After centrifugation at low speed the supernatant was filtered through a 0.22 um filter (Costar, Cambridge, MA). The filtrate (1 ml) was inoculated in Vero E-6 cells for virus isolation. Seven days later, spot-slides were prepared for the Vero E-6 cells and IFA test was performed using mouse positive serum for the detection of the CCHF virus. To-date no virus has been isolated. It may be possible that the Vero E-6 cells that we use are not very sensitive. On the other hand we do not want to use suckling mice for the isolation of the virus because our facilities are not quite proper. This year we are negotiating with

the University Administration to build a P4 contaminant although we don't know how long it will take for the approval to be announced.

## D. COMMENTS

### 1. HFRS

Since starting these investigations in 1981 we have achieved several objectives but we still have more work to do, to understand the epidemiology of the Hantaan virus in Greece.

So far, we have identified several endemic areas, in North and Central Greece, we have described the clinical course of the severe form of the didease as it occurs in Greece, we have isolated the causative agent, we have found a probable host of the virus and recently we have successfully used the ELISA IgM capture for early diagnosis of the disease.

Having done all this, we need to do further work to identify other foci and diagnose the disease in Southern Greece and in the islands, especially those near Turkey. We believe that the disease can not exist only in Northern Greece. We have always had good collaboration and support from hospitals in North and Northwestern Greece, but so far, we have not had much co-operation from institutions in Southern Greece, even though we are the only laboratory able to undertake this work in Greece. We now have a promise from our Ministry of Health and Welfare to persuade the Southern Greek Health Authorities to co-operate with us, and to make use of our facilities. We must trap more small mammals, especially close to the Albanian, Yugoslavian and Bulgarian borders to identify other hosts, and to see whether more strains of Hantaan virus exist in Greece.

We must attempt to discover the reason for the discrepancy between our results in Rodopi and our Bulgarian colleagues' results across the border. We have established good links with Albanian scientists, and hope to help them with their studies; iy would be

beneficial to us if we could have further information about the disease as it occurs in Albania. We need to do further work with the ELISA IgM capture test to establish its specificity.

2.CCHF

As far as CCHF is concerned, we have used the same protocol as for HFRS, but so far have had limited results in spite of a lot of work. We have identified endemic areas, and in some of these areas, especially near Albania and Yugoslavia, the seropositive rate in humans is very high. None of these seropositive individuals has suffered a serious illness in the past, and so far, in spite of good co-operation with hospitals in N. Greece, we have not identified a single clinical case.

We know that several cases were diagnosed in S. Albania last year, and in Bulgaria, where Bulgarian scientists have identified E. Rodopi as high-risk area.

We, therefore, have several theories to explain these differences: the virus isolated in Greece may be antigenically different and may produce sub-clinical infections; we may have missed cases, but this seems unlikely because the clinical disease is severe.

Therefore, we must pursue our attempts to diagnose cases of CCHF. We want to return to a village near Albania, to resample 100 individuals whose blood we examined last year to see if any new seropositives exist. If we find new seropositives we hope that they would be able to remember one year's illnesses and therefore to see if a less serious form of the disease occurs.

We need to collect ticks from endemic areas and try to isolate the virus. We also need to sample blood from sheep and goats in endemic areas to establish their role of seropositivity and therefore the likelihood of an outbreak of the disease in humans.

**E. Publications from April 1987 through April 1988**

1. Clinical and epidemiological aspects of hemmorrhagic fever with renal syndrome (HFRS) in Greece. (Sept. 1987): A. Antoniadis, J.W. LeDuc and S. Alexiou-Daniel. Eur. J. Epidemiol. 3,3.

2. Isolation of a Hantavirus from a severelly ill patient with hemorrhagic fever with renal syndrome in Greece. (Dec. 1987): A. Antoniadis, D. Grekas, C.A. Rossi and J.W. LeDuc. J. Inf. Dis. 156,6.

3. Hemorrhagic Fever with Renal Syndrome in Greece: Clinical and Laboratory Characteristics: A. Antoniadis, J.W. LeDuc, N. Acritidis, S. Alexiou-Daniel, A. Kyparissi and G.A. Saviolakis. Rev. Inf. Dis. In Press.

TABLE 1: HFRS-Human Serosurvey from April 1987 to April 1988

Human Serosurvey

| COUNTY | No. OF SERA EXAMINED | M/F | No. OF POSITIVES1 | M/F |
|---|---|---|---|---|
| Rodope | 140 | 80/ 60 | 1 | 1/- |
| Evros | 117 | 87/ 30 | 1 | 1/- |
| Ioannina | 218 | 117/101 | 6 | 4/2 |
| Halkidiki2 | 24 | 23/ 1 | - | -/- |
| Others3 | 81 | 81/--- | - | -/- |
| TOTAL | 580 | 388/192 | 8 | 6/2 |

1 IFA > 1:16
2 Public Health Veterinarian Institute workers
3 Soldiers serving in Northern Greece

TABLE 2: Serologically Confirmed HFRS Cases (April 1987 through April 1988)

| | NO. OF ALL PATIENTS | HFRS CONFIRMED CASES | DEATHS |
|---|---|---|---|
| Patients with Pyrexia of unknown origin | 111 | - | - |
| Patients suspected of HFRS and Leptospirosis | 46 | 7* | 1* |
| Patients with Hemorrhagic manifestations and Pyrexia | 5 | - | - |
| TOTAL | 202 | 7 | 1 |

* Six patients were residents of Ioannina county and one of Drama county.

Table 3: Serological diagnosis of HFRS in seven patients by IFA and ELISA IgM capture.

| Patients Code No | Day of Onset | IFA IgG | IFA IgM | ELISA IgM |
|---|---|---|---|---|
| 1 | 7th | 1:4096 | 1:2048 | 1:102400 |
| 2 | 10th | 1:2048 | 1: 256 | 1: 51200 |
| 3 | 8th | 1:8192 | 1:2048 | 1: 25600 |
| 4 | 6th | 1:1024 | 1:4096 | 1:816000 |
| 5 | 6th | 1:1024 | 1:2048 | 1:102400 |
| 6 | 12th | 1:8192 | 1:1024 | 1:102400 |
| 7 | 8th | 1:4096 | 1:1024 | 1: 51200 |

Table 4. Clinical findings in 27 Greek patients with hemorrhagic fever with renal syndrome.

| Symptoms | No. of Patients | Signs | No. of patients |
|---|---|---|---|
| Fever | 27 | Renal insufficiency | |
| Rigors | 27 | (oliguria or anuria) | 27 |
| Headache | 27 | Hypotension | 16 |
| Severe malaise | 23 | Flushing of face and neck | 16 |
| Abdominal pain | 23 | Conjunctival injection | 16 |
| Myalgia | 21 | Confusion | 12 |
| Vomiting | 21 | Precoma or coma | 12 |
| Back pain | 19 | Shock | 8 |
| Arthralgia | 8 | Hemorrhages | 6 |
| Diarrhea | 6 | Pulmonary infiltrates | 4 |
| Cough | 3 | Pulmonary edema | 4 |

Table 5. Clinical and laboratory findings in six patients with hemorrhagic manifestations.

| | Patient | | | | | |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | F |
| **Clinical signs** | | | | | | |
| Petechiae | + | – | – | – | + | + |
| Nasal bleeding | + | – | – | – | + | + |
| Gastrointestinal bleeding | – | + | + | – | + | + |
| Hematuria | + | + | – | + | – | – |
| **Laboratory data** | | | | | | |
| Hematocrit (%) | 58 | 59 | 59 | 52 | 51 | 58 |
| WBC (cells/μL) | 14,800 | 11,900 | 21,200 | 20,100 | 11,400 | 17,100 |
| Platelets/μL | 35,000 | 75,000 | 113,000 | 80,000 | 40,000 | 50,000 |
| Prothrombin time (s) | 17 | NA | NA | 14 | 18 | 17 |
| Partial thromboplastin time (s) | 53 | NA | NA | 18 | 36 | 42 |
| Fibrin-fibrinogen degradation products (μg/mL) | 160 | NA | NA | NA | 110 | 54 |

NOTE. + = present; – = not present; NA = not available.

TABLE 6. SEROLOGICAL CONFIRMED HFRS CASES LIVING IN DIFFERENT COUNTIES.

| COUNTRIES | No of cases | M/F | No of deads |
|---|---|---|---|
| 1. IOANNINA | 27 | (23/4) | 6 |
| 2. PELLA | 5 | ( 4/1) | - |
| 3. KARDITSA | 1 | ( 1/-) | - |
| 4. KOZANI | 1 | ( 1/-) | - |
| 5. KATERINI | 2 | ( 2/-) | - |
| 6. KASTORIA | 1 | ( 1/-) | - |
| 7. KILKIS | 1 | ( 1/-) | 1 |
| 8. DRAMA | 1 | ( 1/-) | - |
| 9. SERRES | 1 | ( 1/-) | - |
| 10. TRIKALA | 1 | ( 1/-) | - |
| TOTAL | 41 | 36/5 | 7 |

RATIO FEMALE MALE
F:5 M:36

TABLE 7: Small Rodents Captured in HFRS Endemic and non Endemic Areas

| COUNTY | NO. OF RODENTS | NO. OF POSITIVES | TITERS |
|---|---|---|---|
| Ioannina | 15 Ap. flavicollis | 1 | 1: 64 |
| | + 3 Ap. sylvaticus | - | |
| | 18 | | |
| Pella | 10 Ap. flavicollis | - | |
| | 2 Ap. sylvaticus | - | |
| | + 1 Unidentified | - | |
| | 13 | | |
| Drama | 4 Ap. flavicollis | 1 | 1:128 |
| | + 1 Ap. sylvaticus | - | |
| | 5 | | |
| Thessaloniki | 18 Rattus sp. | - | |
| TOTAL | 54 | 2 | |

TABLE 8 — Small mammals captured in endemic and nonendemic areas in Greece tested for IFA antibodies to Hantaan virus.

| Location | Species | No. of trapped | No. of positives* |
|---|---|---|---|
| Region: Epirus | *Rattus rattus alexandrinus* | 41 | — |
| County: Ioannina * | *R. rattus frugivorus* | 10 | — |
| Area: Tsepelovo | *Apodemus flavicollis* | 23 | 2 |
| | *A. sylvaticus* | 1 | |
| | *Crocidura* sp. | 1 | — |
| Region: Macedonia | | | |
| County: Pella * | | | |
| Area: Promahi | *Apodemus flavicollis* | 19 | 2 |
| | *A. sylvaticus* | 4 | — |
| | *Mus domesticus* | 1 | — |
| County: Serres ** | | | |
| Area: Rice fields | *Apodemus flavicollis* | 9 | — |
| County: Thessaloniki | | | |
| Area: Rice fields | *Apodemus sylvaticus* | 3 | — |
| | *A. flavicollis* | 9 | — |
| County: Thessaloniki ** | | | |
| Area: Slaughter house | *Rattus rattus alexandrinus* | 15 | 2 |
| Total | | 136 | 6 |

* Endemic «high risk» areas.
** Endemic areas.
*IFA titers ranging from 1:64 to 1:2048.

**Table 9.** PRN tests with Hantaan virus, Seoul virus, Puumala virus, and the virus from our patient with HFRS, and a comparison of antibody titers to Hantaan virus and the Greek isolate with sera from previously diagnosed Greek patients with HFRS.

| Sera | Antibody titers to | | | |
|---|---|---|---|---|
| | Hantaan virus | Seoul virus | Puumala virus | Greek isolate |
| Immune sera from patient with KHF | 2,048 | <8 | 64 | 256 |
| Rat antisera to Seoul virus | 256 | 2,048 | <16 | 1,024 |
| Immune sera from patient with NE | <8 | <8 | 1,024 | 16 |
| Sera from patient studied | | | | |
| Day 14 | 1,024 | <8 | 32 | 1,024 |
| Day 24 | 32 | <8 | <8 | 512 |
| Sera from previously diagnosed patients | | | | |
| Patient 3 | | | | |
| Day 8 | 64 | NT | NT | 512 |
| Day 106 | 128 | NT | NT | 2,048 |
| Patient 8 | | | | |
| Day 14 | 1,024 | NT | NT | 2,048 |
| Day 24 | 32 | NT | NT | 512 |
| Patient 13 | | | | |
| Day 20 | 128 | NT | NT | 2,048 |
| Day 35 | 256 | NT | NT | 512 |
| Patient 15, day 28 | 256 | NT | NT | 256 |
| Patient 18, day 10 | 1,024 | NT | NT | 2,048 |

NOTE. Data are the reciprocal of the highest dilution neutralizing 50% of the plaque dose (~100 pfu). NT, not tested.

Table 10. Comparison of titers obtained by immunofluorescent antibody (IFA), assay and IgM capture enzyme immunoassay (EIA) on the first serum sample submitted for serological confirmation of HFRS.

| Patient No | Day post-onset | IFA* | EIA |
|---|---|---|---|
| 1 | 5 | 256 | 408000 |
| 2 | 6 | 2048 | 204000 |
| 3 | 6 | 256 | 204000 |
| 4 | 6 | 1024 | 816000 |
| 5 | 7 | 8192 | 408000 |
| 6 | 7 | 512 | 25600 |
| 7 | 7 | 1024 | 102000 |
| 8 | 8 | 2048 | 51200 |
| 9 | 8 | 256 | 25600 |
| 10 | 8 | 2048 | 51200 |
| 11 | 8 | 2048 | 408000 |
| 12 | 8 | 1024 | 816000 |
| 13 | 8 | 256 | 51200 |
| 14 | 8 | 512 | 102400 |
| 15 | 9 | 1024 | 816000 |
| 16 | 10 | 512 | 102400 |
| 17 | 10 | 256 | 102400 |
| 18 | 10 | 4096 | 204000 |
| 19 | 10 | 1024 | 816000 |

* anti-human IgM conjugate

TABLE 11: CCHF Human Serosurvey from April 1987 through April 1988

| NO. OF SERA EXAMINED | COUNTY | POSITIVES* | M/F | TITERS |
|---|---|---|---|---|
| 140 | Rodopi | 1 | 1/- | 1:8 (1) |
| 117 | Evros | - | -/- | |
| 218 | Ioannina | 5 | 4/1 | 1:16 (2)<br>1:32 (3) |
| 24 | Veterinarians | 1 | 1/- | 1:32 (1) |
| 80 | Soldiers | - | -/- | |
| 579 TOTAL | | 7 | 6/1 | |

* IFA > 1:16

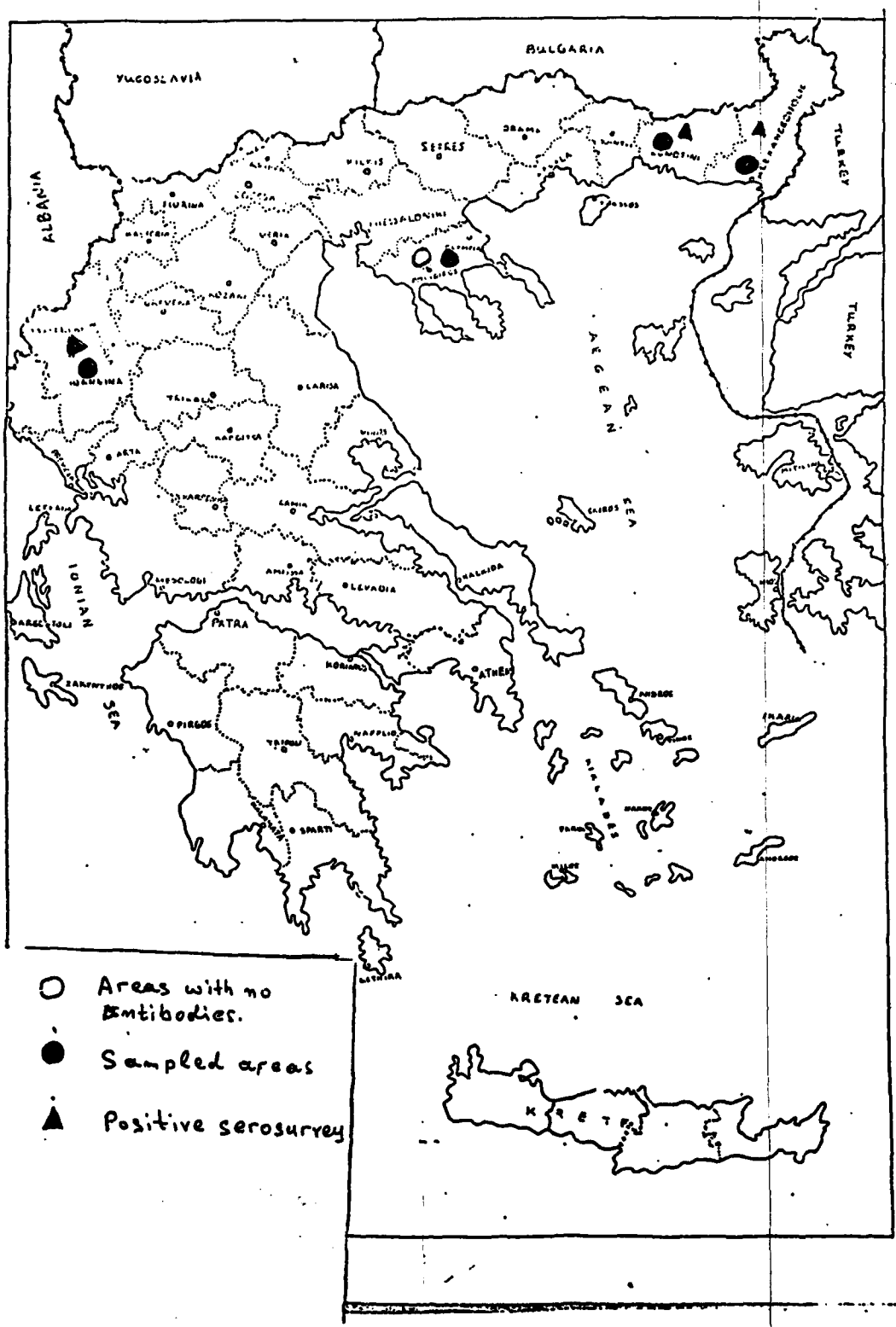
TABLE 12: PATIENTS EXAMINED FOR CCHF INFECTION

| | |
|---|---|
| Patients with Pyrexia of unknown origin | 111 |
| Patients resembling CCHF (Hemorrhagic manifestations, fever) | 16 |
| Patients with elevated liver enzymes, negative for Hepatitis A and B, CMV and EB | 72 |
| TOTAL | 169 |

TABLE 13 : Tick pools collected from April 1987 throught April 1988

| Tick species | Number of pools | Animal species |
|---|---|---|
| Rhipicephalus sanguineus | 3 | Goat |
| Ixodes gibbosus | 7 | Goat |
| Rhipicephalus bursa | 6 | Goat-sheep |
| Hyalomma anat. anatolicum | 7 | Goat-sheep |
| Unidentified | 6 | Goat |
| | 29 | |

Fig 4 Map of Greece indicating the counties where serosurvey was conducted



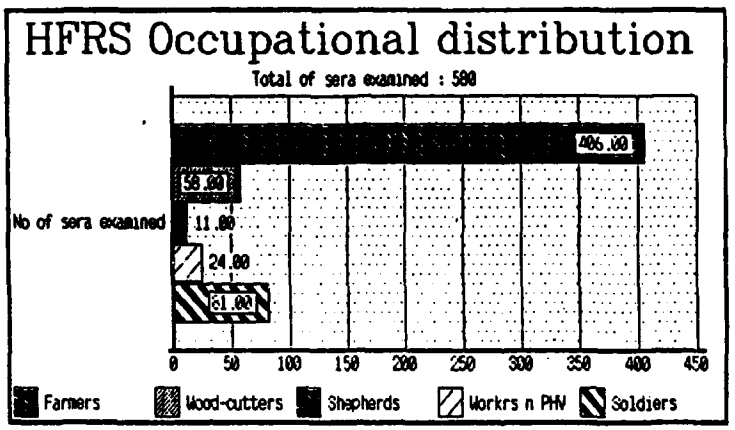

HFRS Occupational distribution
Total of sera examined : 580
No of sera examined
406.00
58.00
11.00
24.00
61.00
0
50
100
150
200
250
300
350
400
450
Farmers
Wood-cutters
Shapherds
Workrs n PHV
Soldiers

Figure 2.

Figure 3.

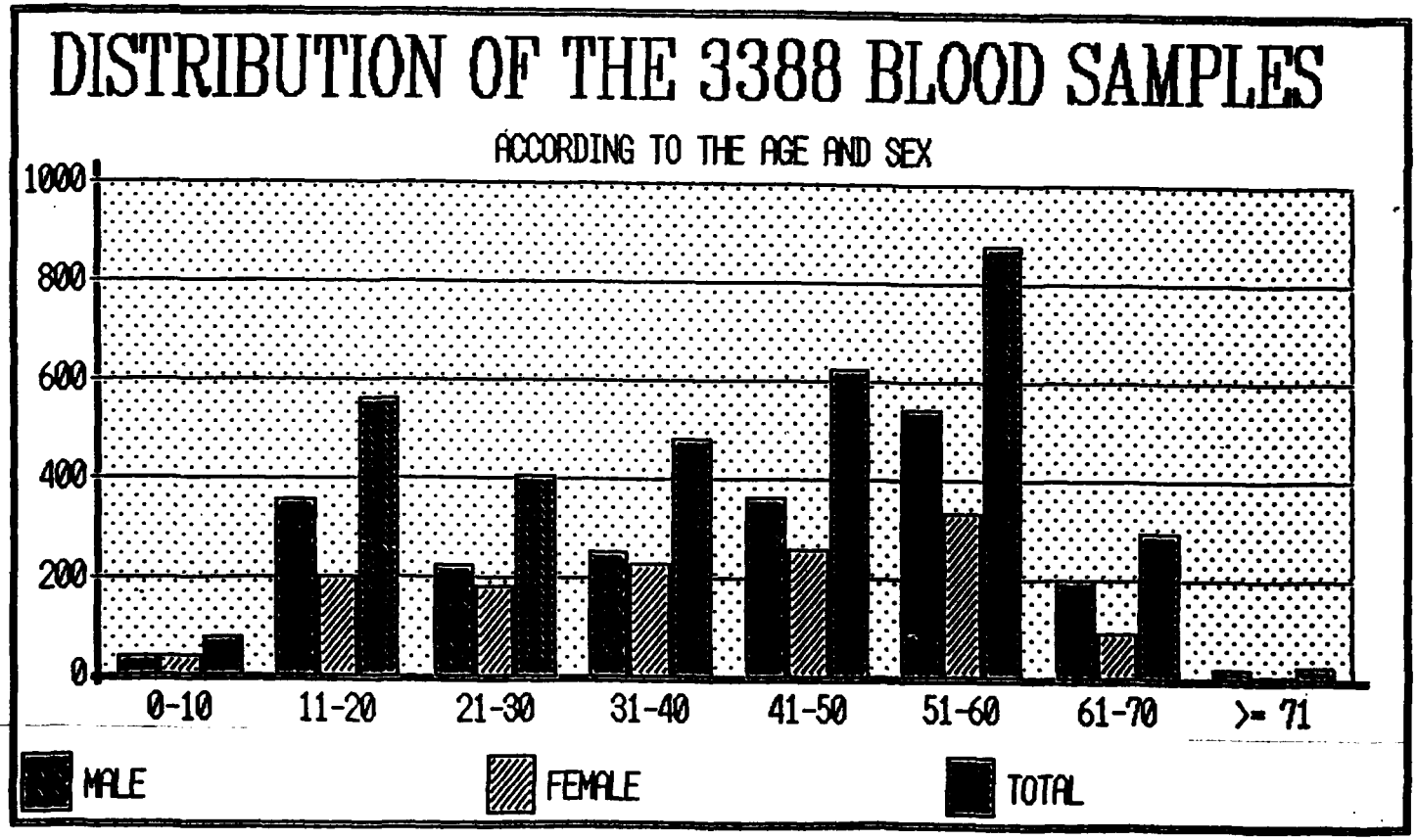

DISTRIBUTION OF THE 3388 BLOOD SAMPLES
ACCORDING TO THE AGE AND SEX
1000
800
600
400
200
0
0-10
11-20
21-30
31-40
41-50
51-60
61-70
>= 71
MALE
FEMALE
TOTAL

Figure 4.

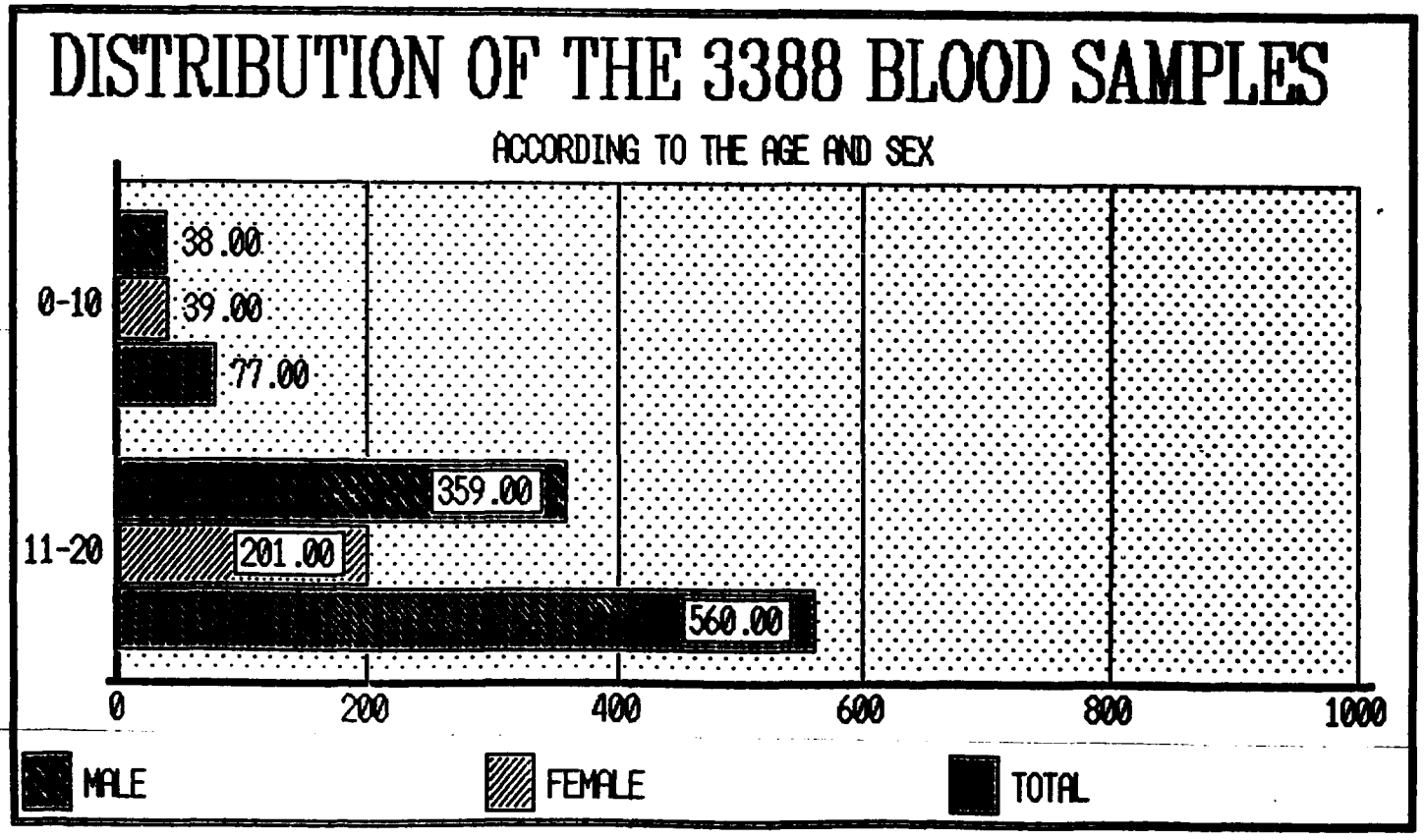

DISTRIBUTION OF THE 3388 BLOOD SAMPLES
ACCORDING TO THE AGE AND SEX
0-10
11-20
38.00
39.00
77.00
359.00
201.00
560.00
0
200
400
600
800
1000
MALE
FEMALE
TOTAL

Figure 5.

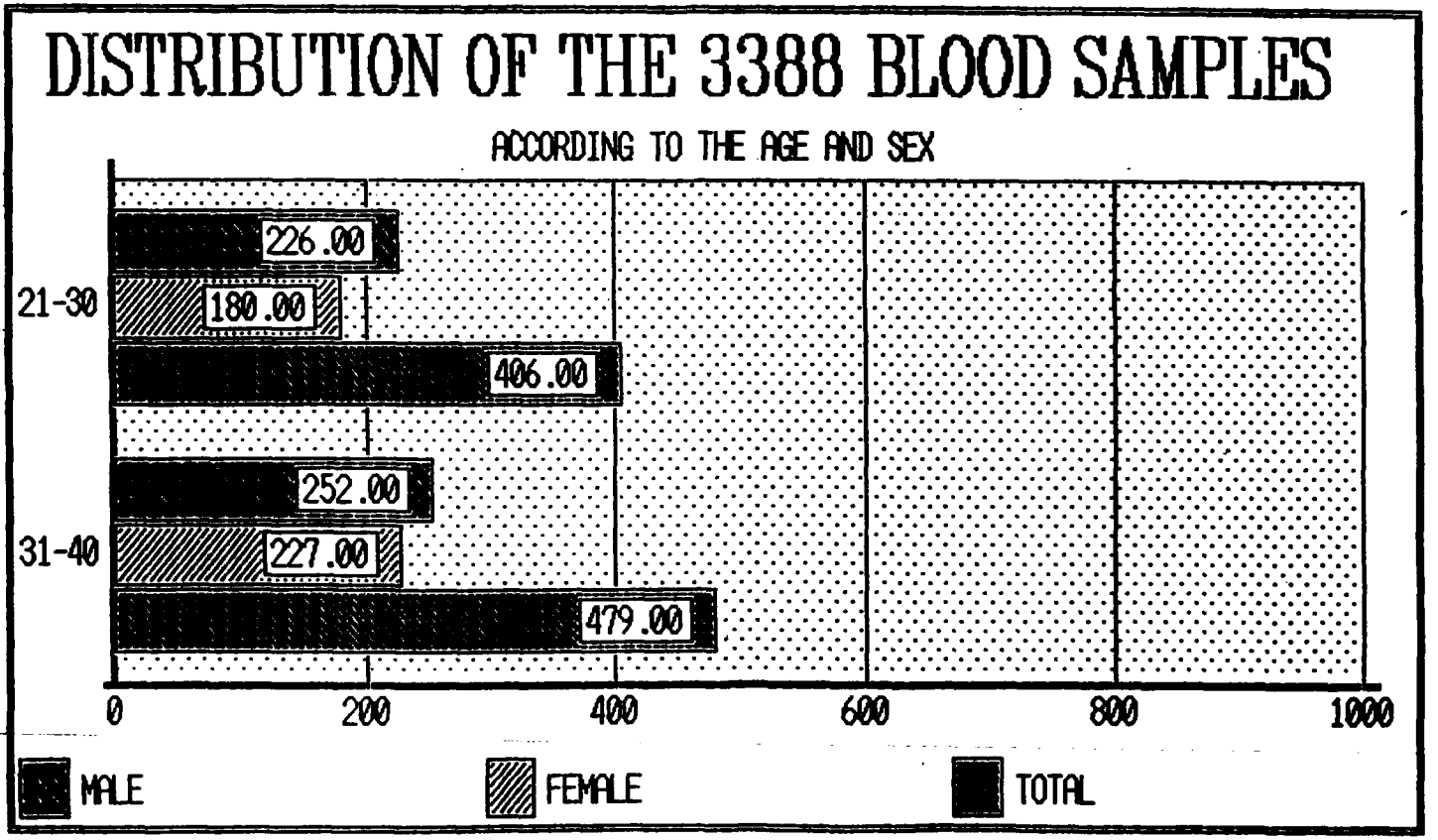

DISTRIBUTION OF THE 3388 BLOOD SAMPLES
ACCORDING TO THE AGE AND SEX
21-30
226.00
180.00
406.00
31-40
252.00
227.00
479.00
0
200
400
600
800
1000
MALE
FEMALE
TOTAL

Figure 6.

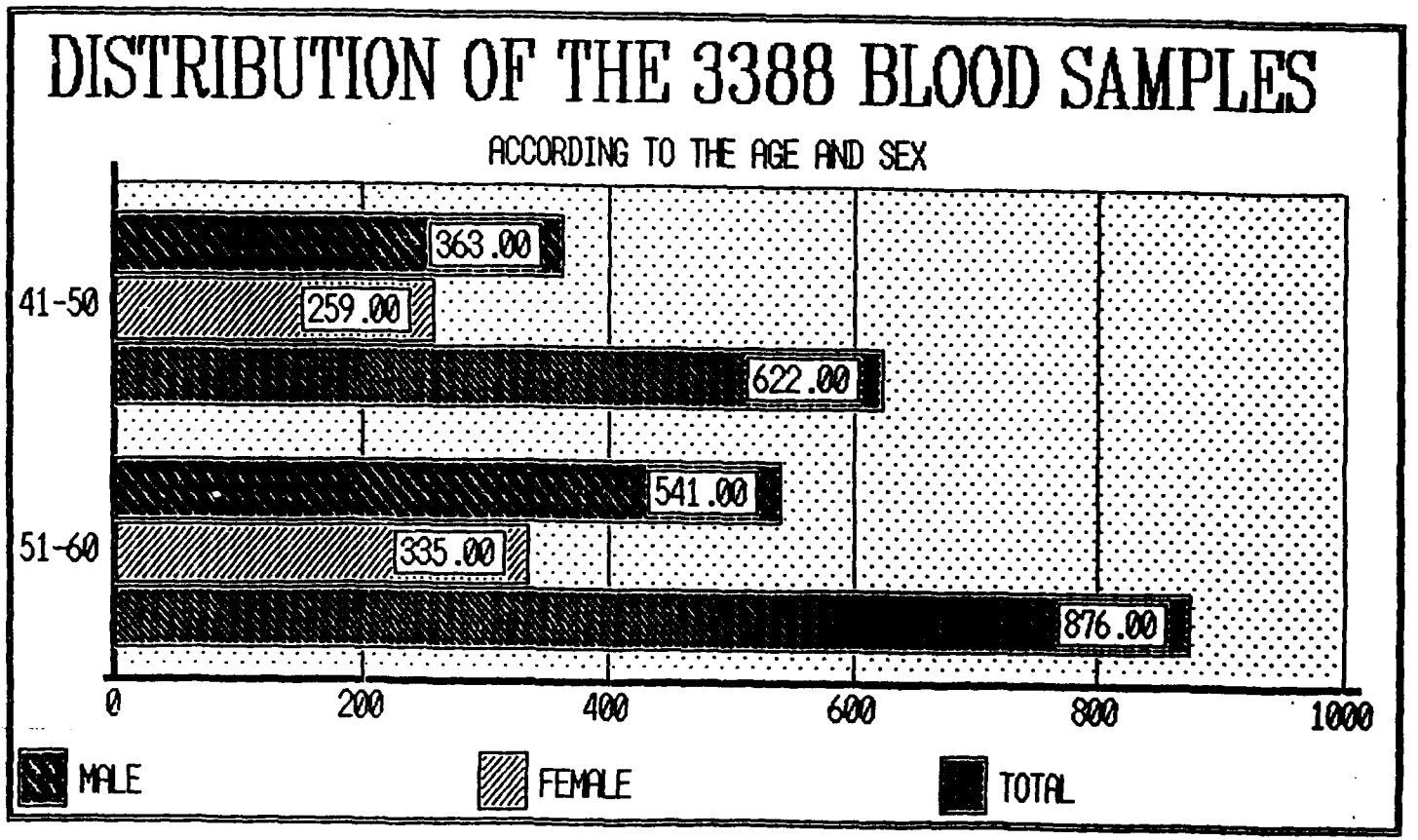

DISTRIBUTION OF THE 3388 BLOOD SAMPLES
ACCORDING TO THE AGE AND SEX
41-50
51-60
363.00
259.00
622.00
541.00
335.00
876.00
0
200
400
600
800
1000
MALE
FEMALE
TOTAL

Figure 7.

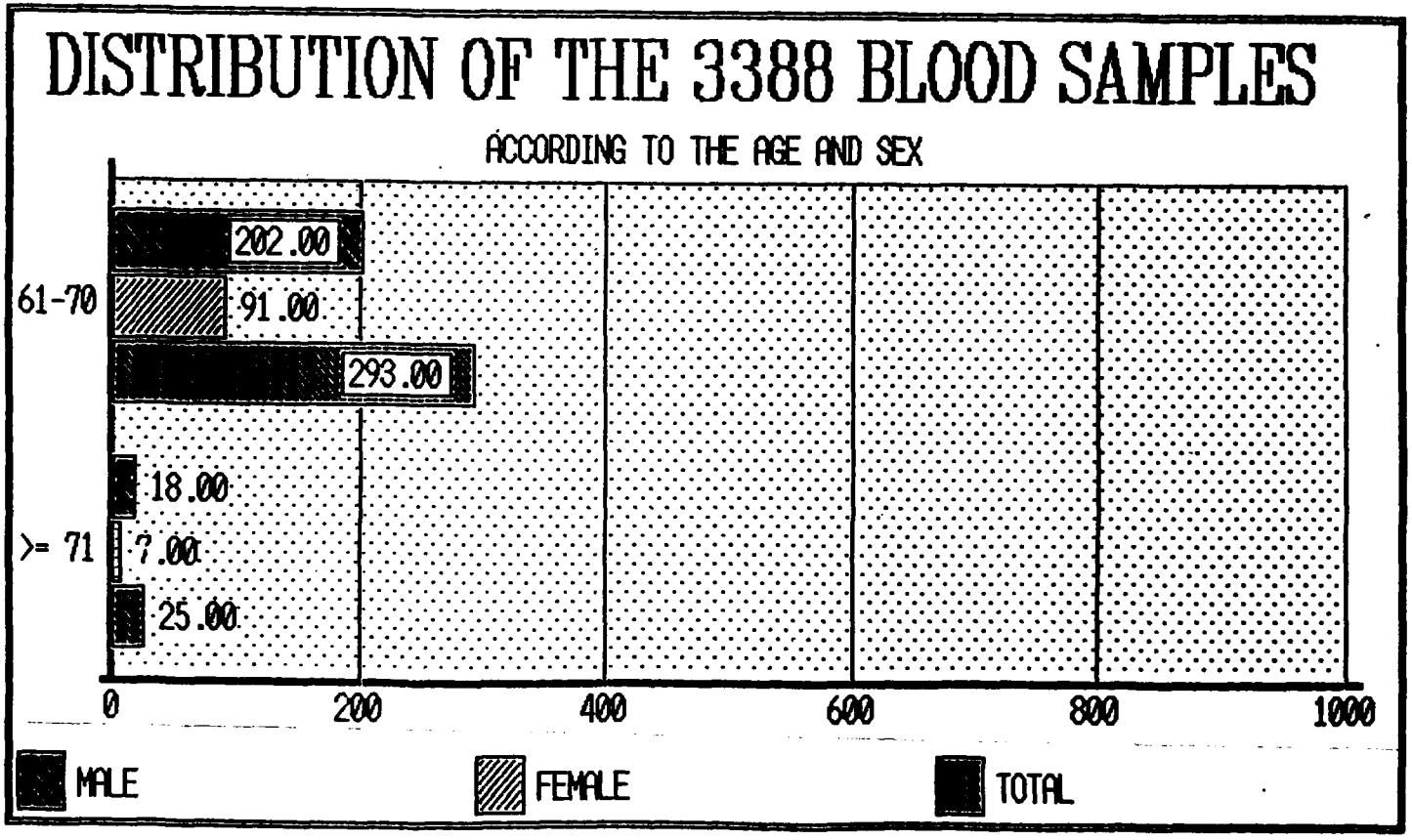

DISTRIBUTION OF THE 3388 BLOOD SAMPLES
ACCORDING TO THE AGE AND SEX
61-70
>= 71
202.00
91.00
293.00
18.00
7.00
25.00
0
200
400
600
800
1000
MALE
FEMALE
TOTAL

Figure 8.

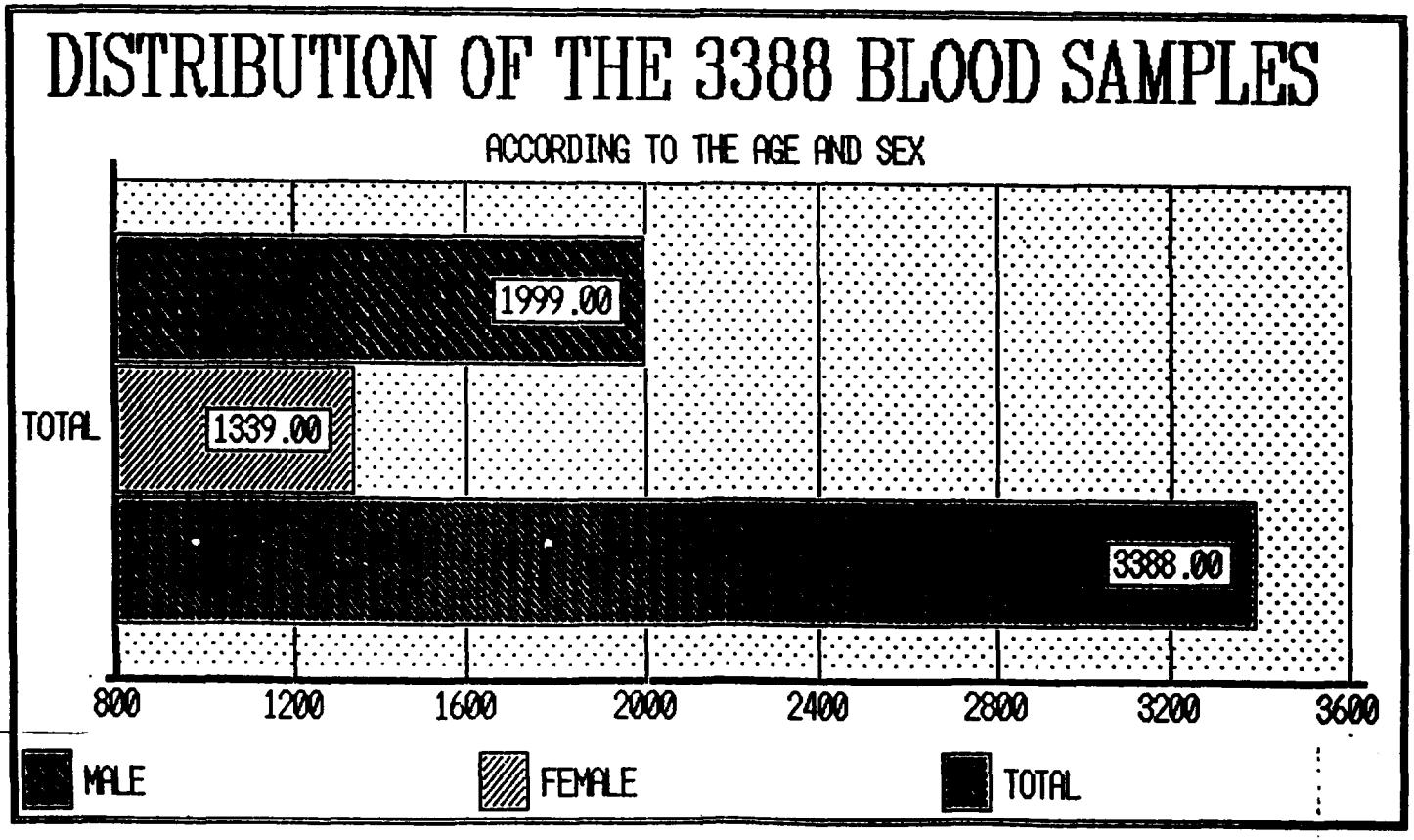

DISTRIBUTION OF THE 3388 BLOOD SAMPLES
ACCORDING TO THE AGE AND SEX
1999.00
TOTAL
1339.00
3388.00
800
1200
1600
2000
2400
2800
3200
3600
MALE
FEMALE
TOTAL

Figure 9. Map of Greece indicating the counties where seropositives were identified

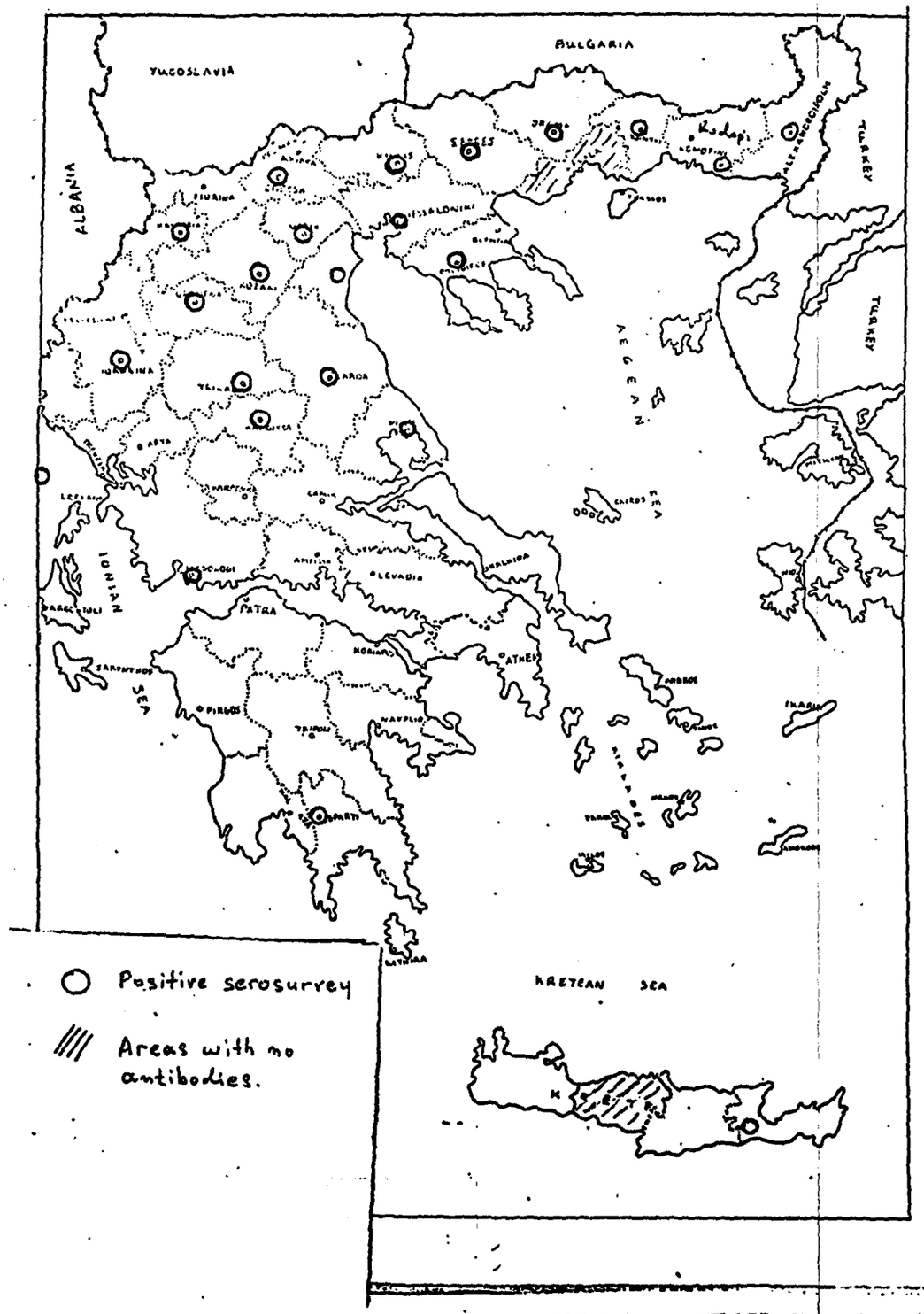
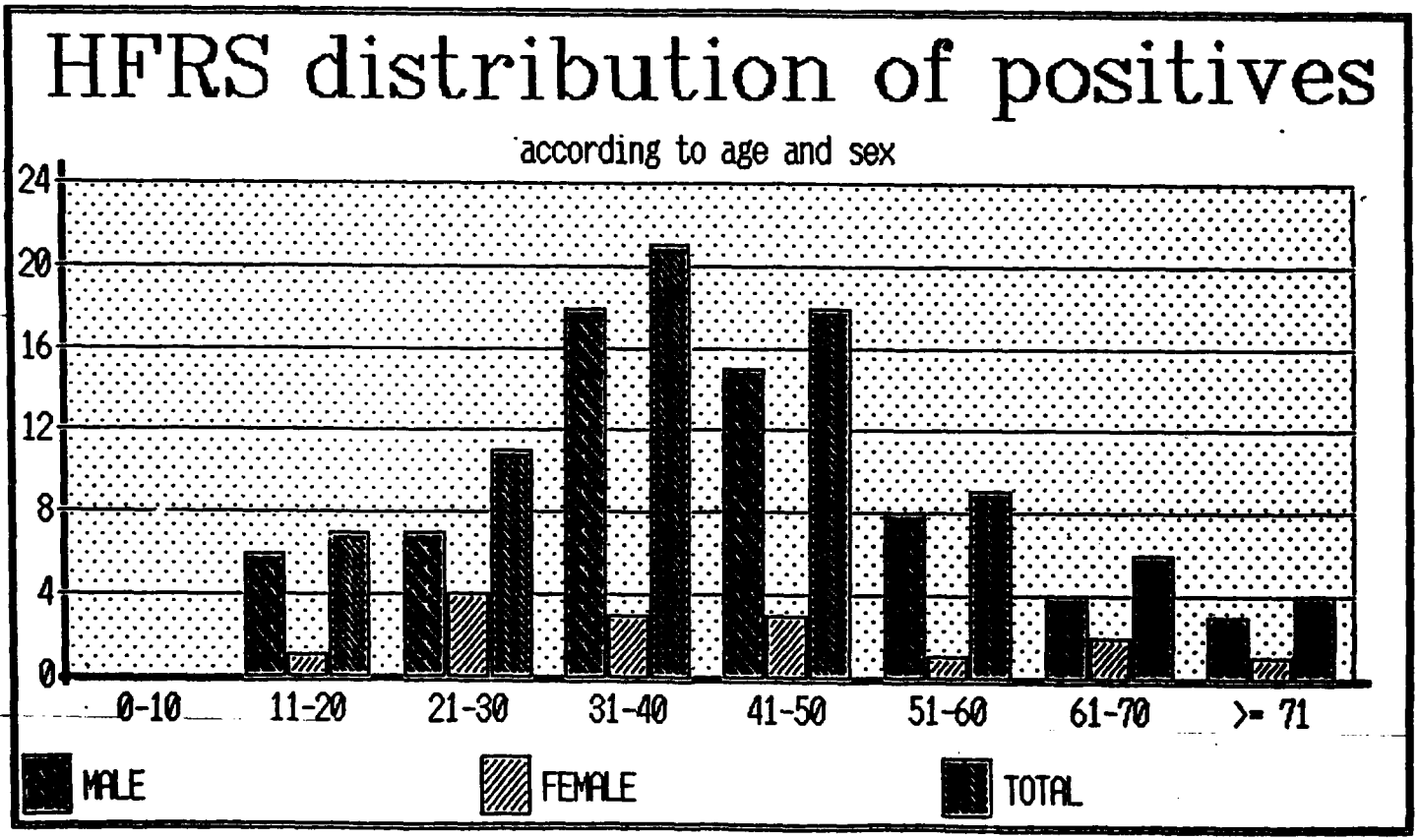


Figure 10.


HFRS distribution of positives
according to age and sex
24
20
16
12
8
4
0
0-10
11-20
21-30
31-40
41-50
51-60
61-70
>= 71
MALE
FEMALE
TOTAL

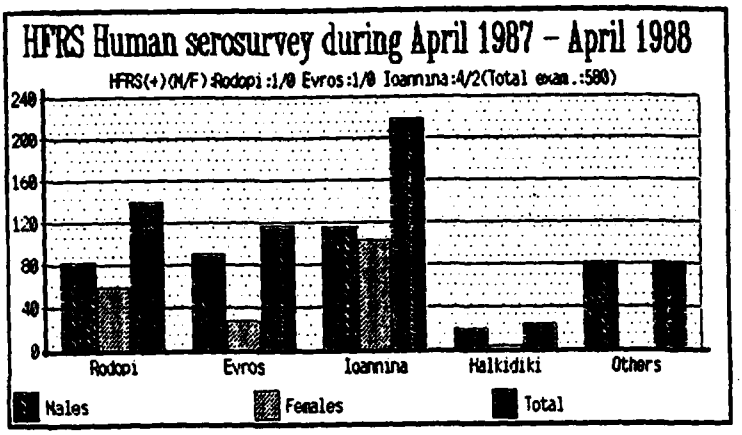

HFRS Human serosurvey during April 1987 - April 1988
HFRS(+)(M/F):Rodopi:1/0 Evros:1/0 Ioannina:4/2(Total exam.:580)
240
200
160
120
80
40
0
Rodopi
Evros
Ioannina
Halkidiki
Others
Males
Females
Total

Fig. 11

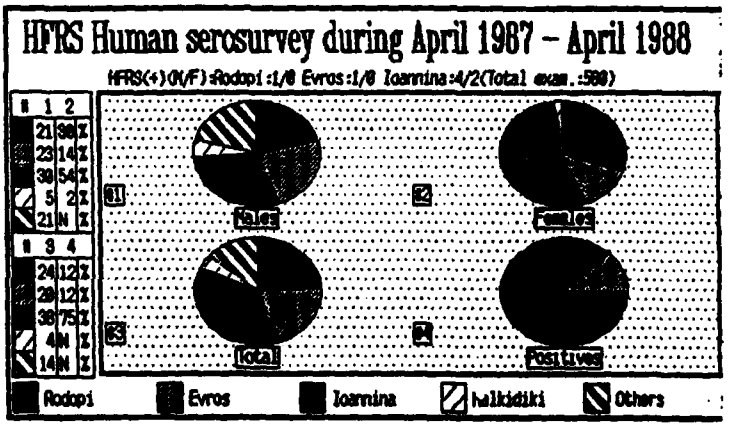

HFRS Human serosurvey during April 1987 - April 1988
HFRS(+)(M/F):Rodopi:1/0 Evros:1/0 Ioannina:4/2(Total exam.:580)
# 1 2
21 38 %
23 14 %
38 54 %
5 2 %
21 N %
# 3 4
24 12 %
28 12 %
38 75 %
4 N %
14 N %
Males
Females
Total
Positives
Rodopi
Evros
Ioannina
Halkidiki
Others

Fig. 12

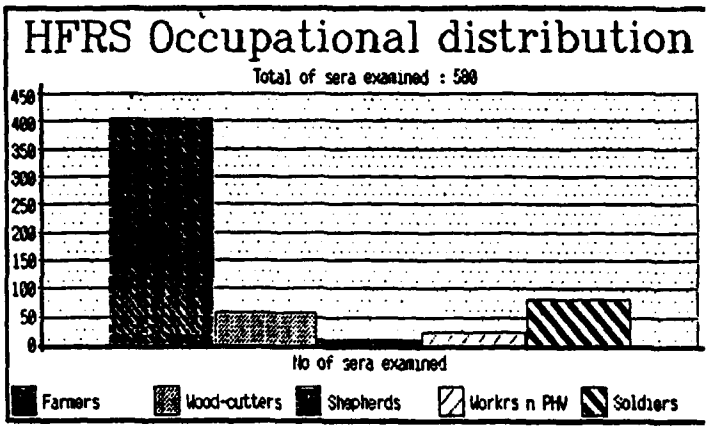



Figure 13.

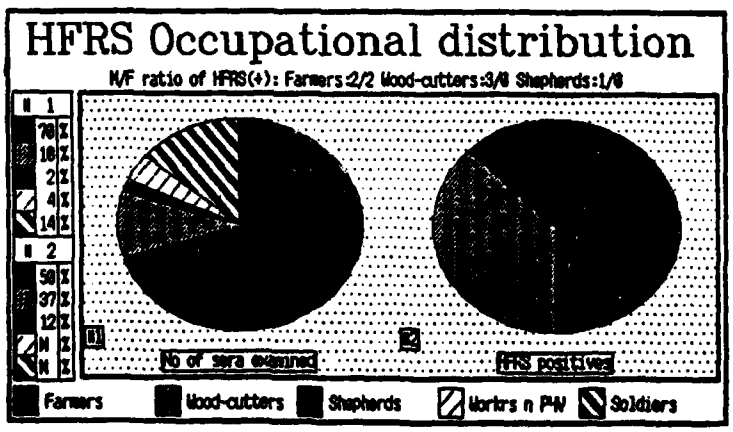
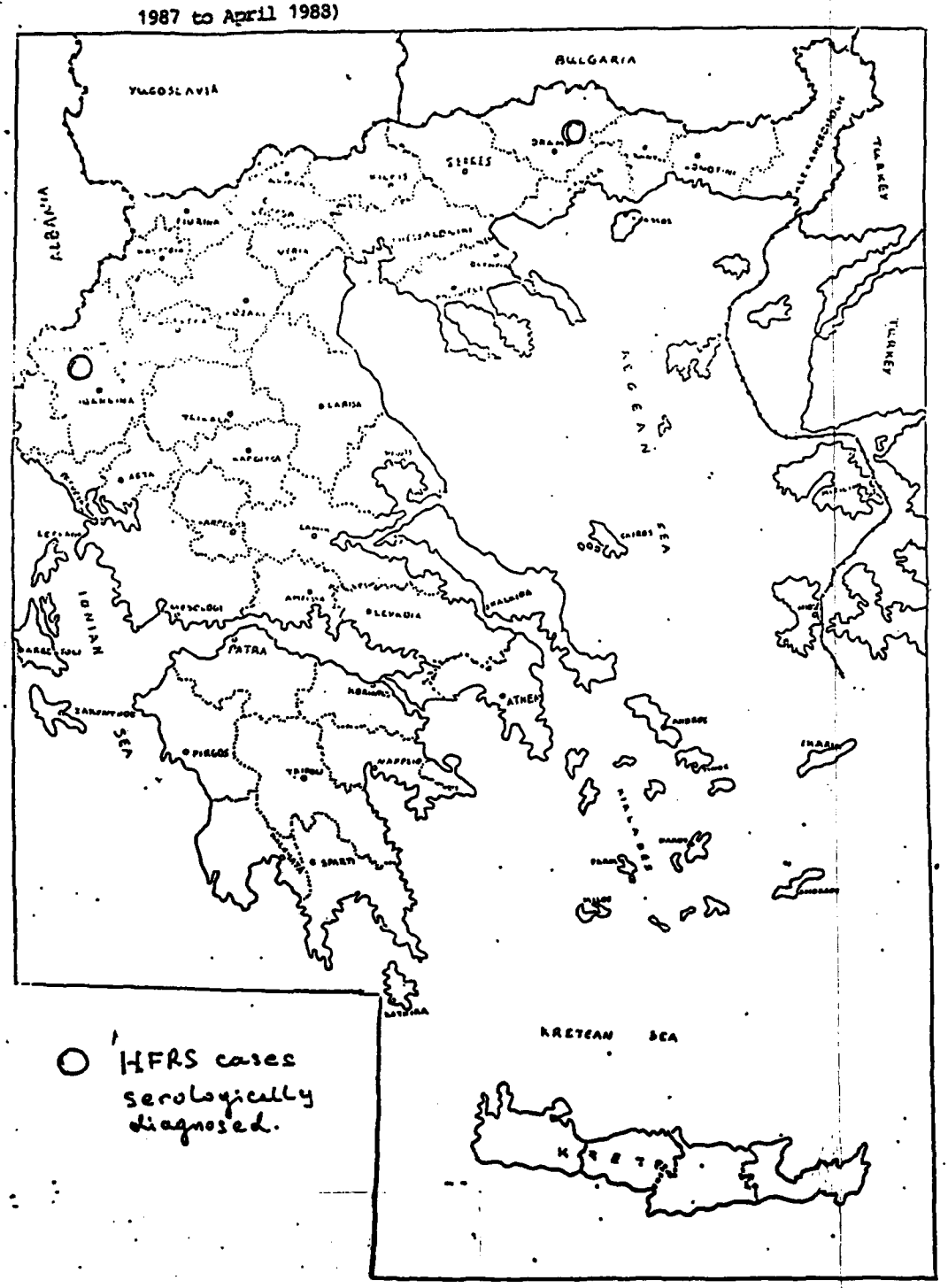



Figure 14.

Figure 15. Counties where HFRS cases have been serologically diagnosed (From April 1987 to April 1988)



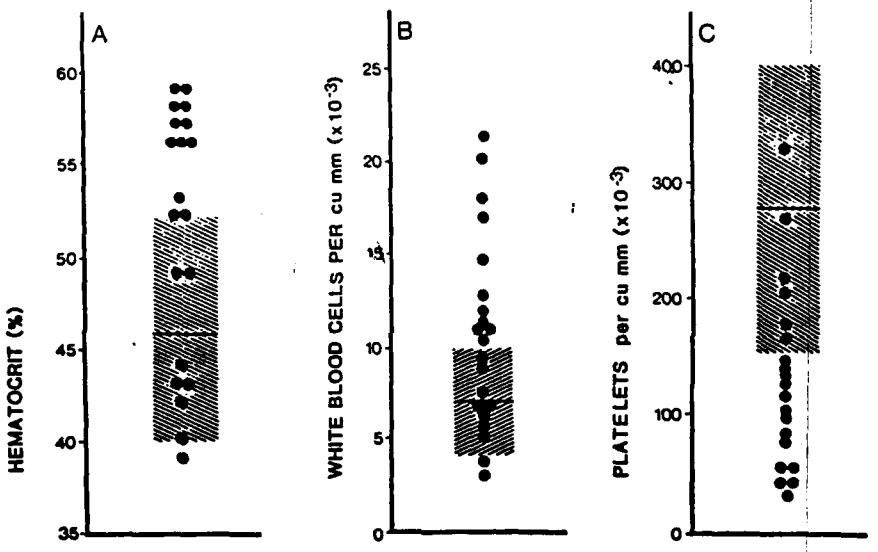



Figure 16. Hematocrit (A) and blood cell counts (B and C) in patients with hemorrhagic fever with renal syndrome (A)=patient data (mean ± SD) obtained between the fifth and seventh day of illness. Shaded area indicates normal laboratory reference values (mean ± SD).

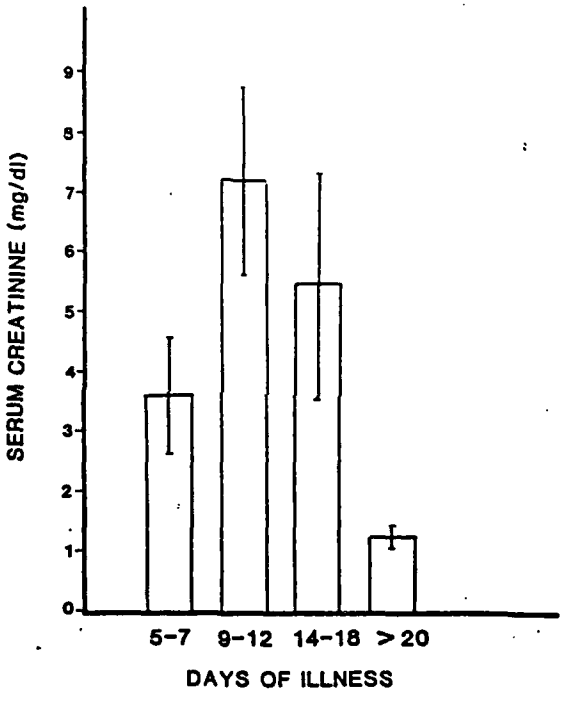



Figure 17. Time course of changes in serum creatinine levels in patients with hemorrhagic fever with renal syndrome. Data shown are mean + SD for 15 patients.

Figure 18.

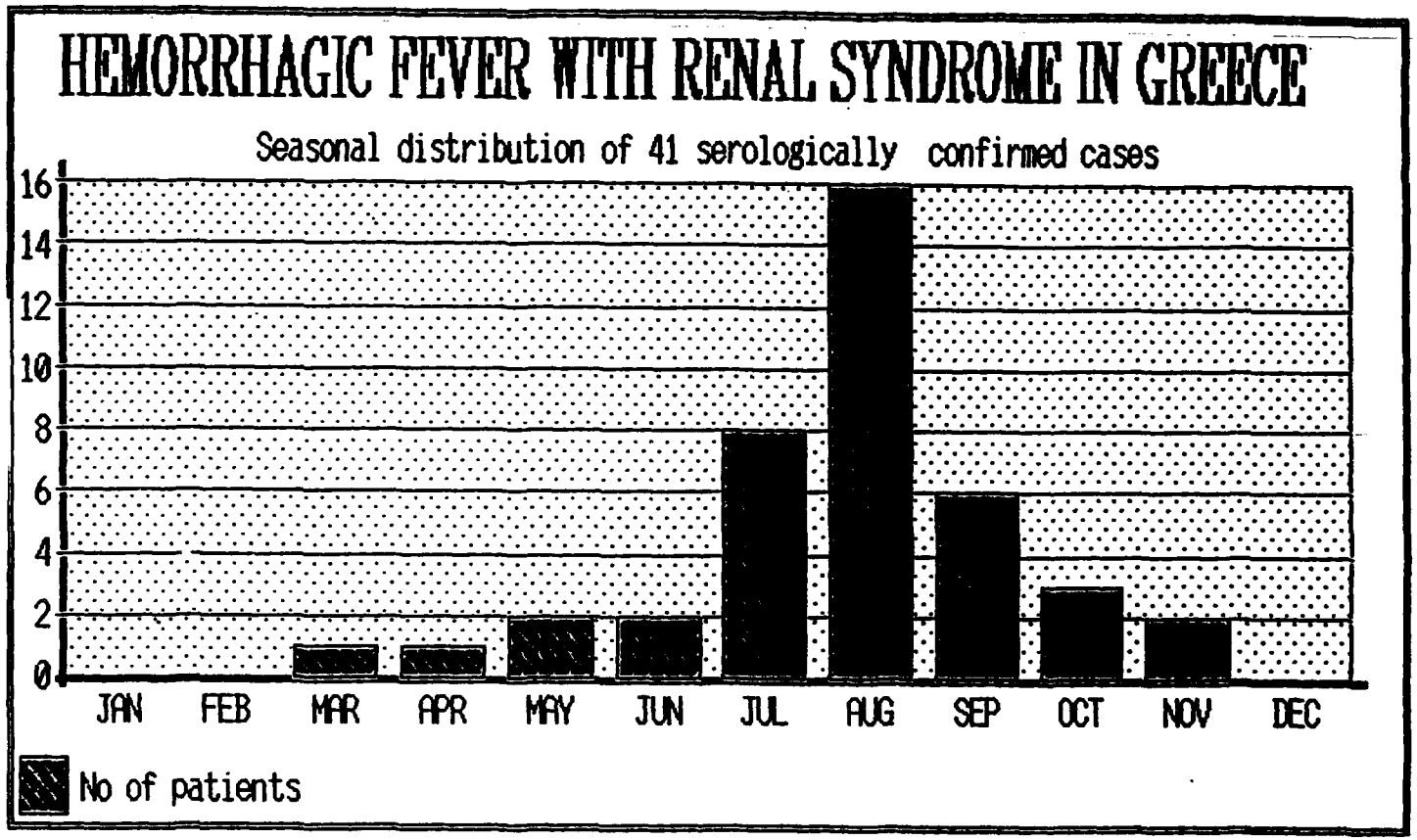

HEMORRHAGIC FEVER WITH RENAL SYNDROME IN GREECE
Seasonal distribution of 41 serologically confirmed cases
16
14
12
10
8
6
4
2
0
JAN
FEB
MAR
APR
MAY
JUN
JUL
AUG
SEP
OCT
NOV
DEC
No of patients

Figure 19. Map of Greece indicating the counties where HFRS cases have been serologically diagnosed

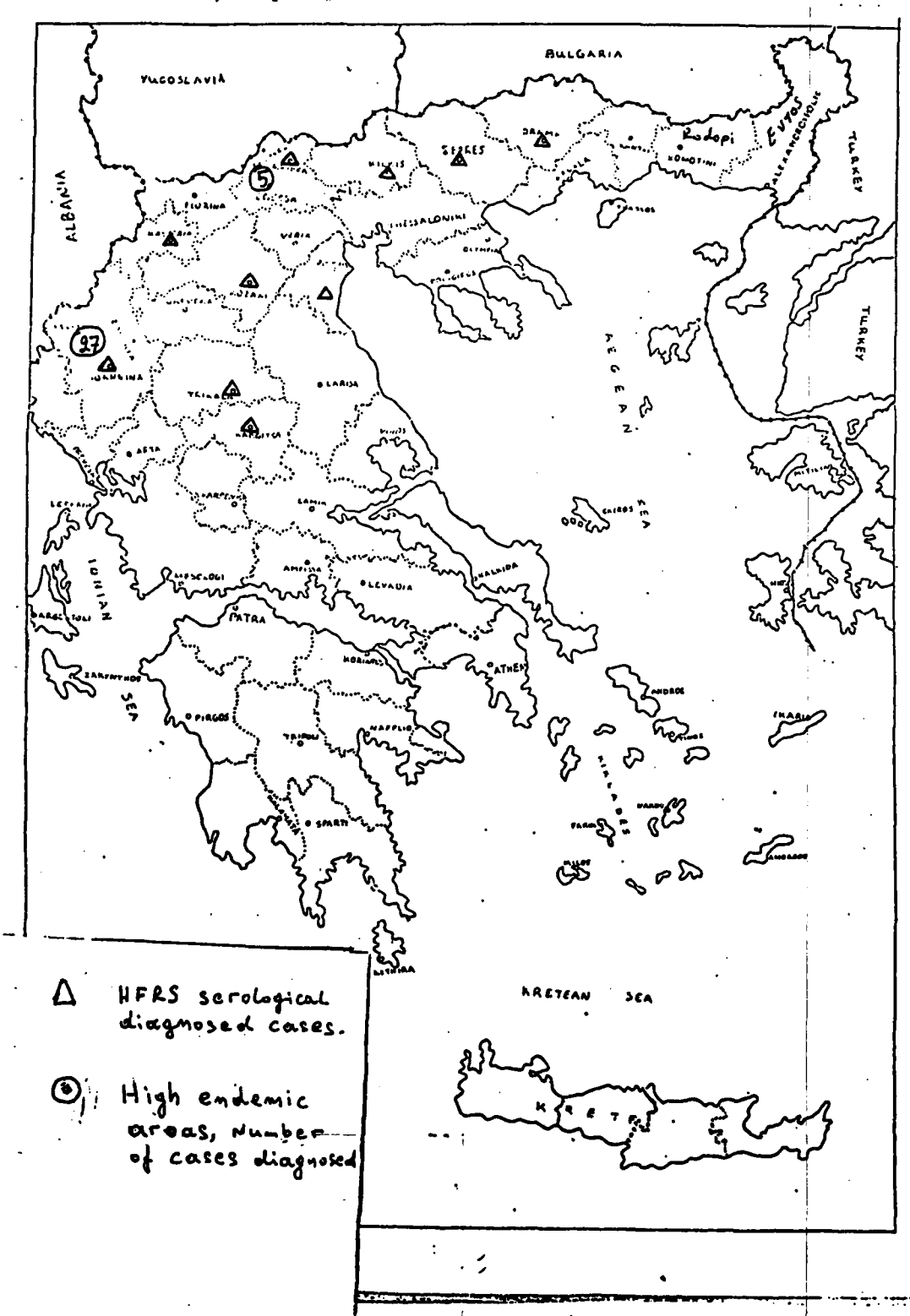


Figure 20.

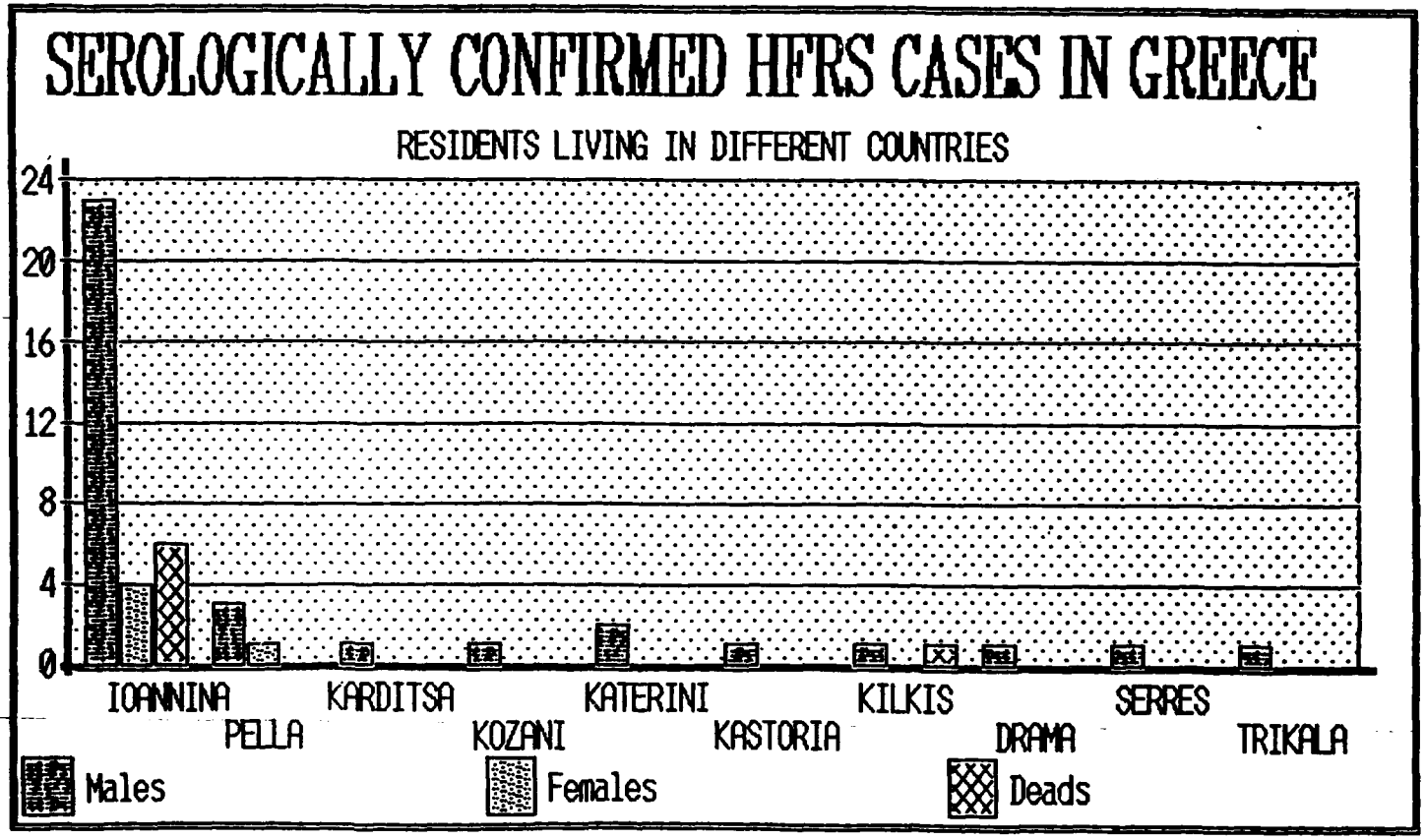

SEROLOGICALLY CONFIRMED HFRS CASES IN GREECE
RESIDENTS LIVING IN DIFFERENT COUNTRIES
24
20
16
12
8
4
0
IOANNINA
PELLA
KARDITSA
KOZANI
KATERINI
KASTORIA
KILKIS
DRAMA
SERRES
TRIKALA
Males
Females
Deads

Figure 21.

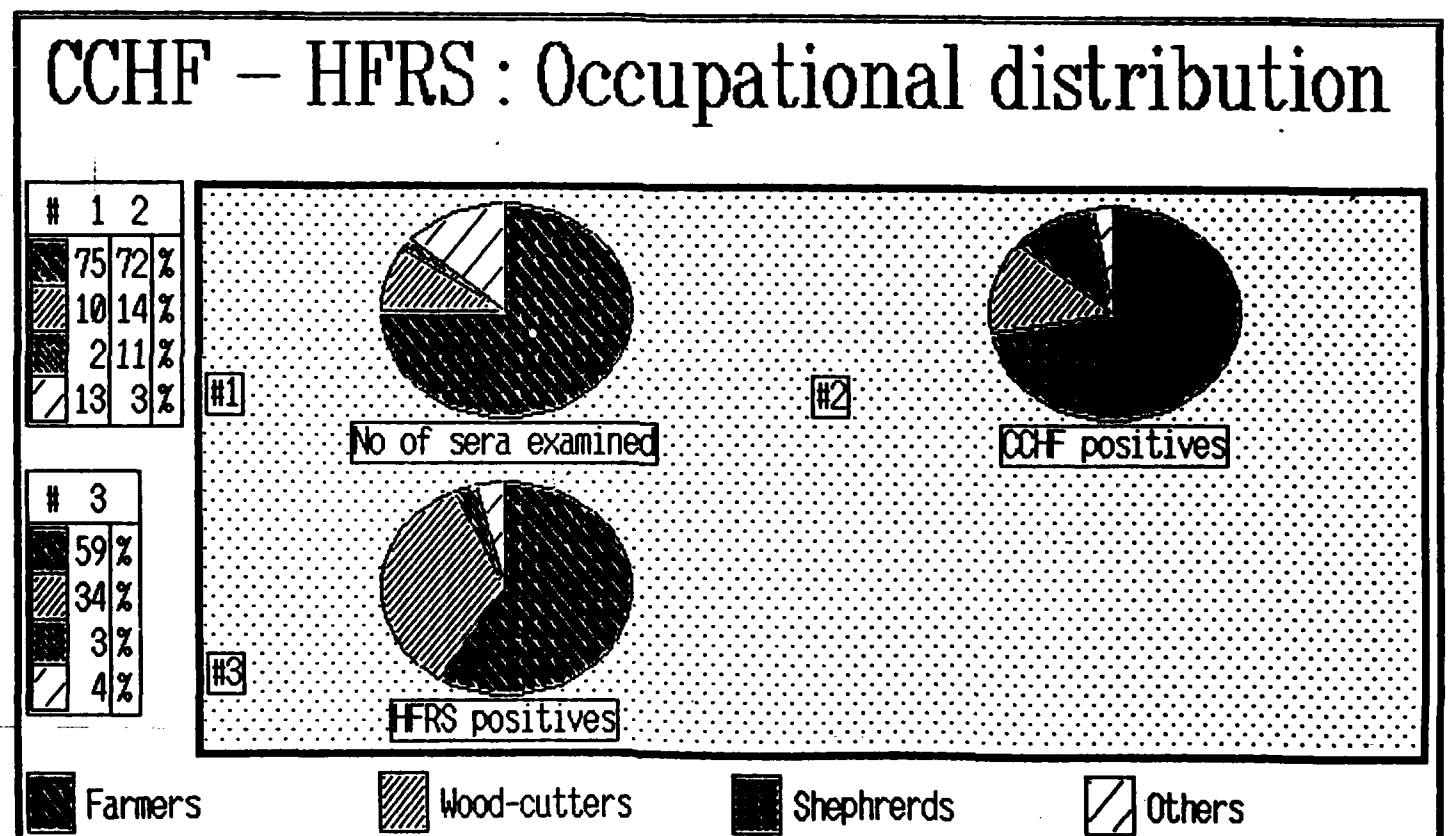
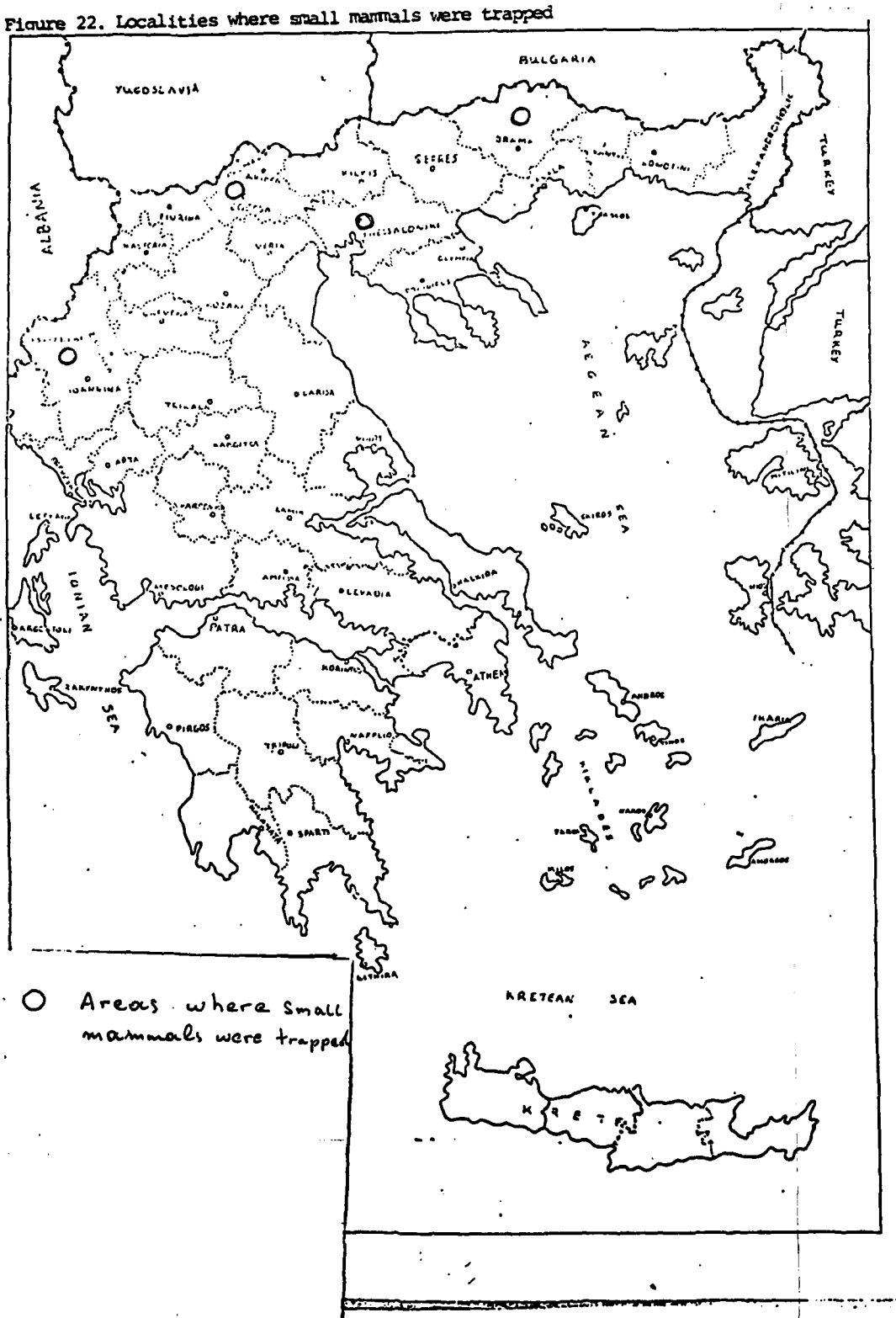

CCHF – HFRS : Occupational distribution
# 1 2
75 72 %
10 14 %
2 11 %
13 3 %
# 3
59 %
34 %
3 %
4 %
#1
No of sera examined
#2
CCHF positives
#3
HFRS positives
Farmers
Wood-cutters
Shephrerds
Others

Figure 22. Localities where small mammals were trapped



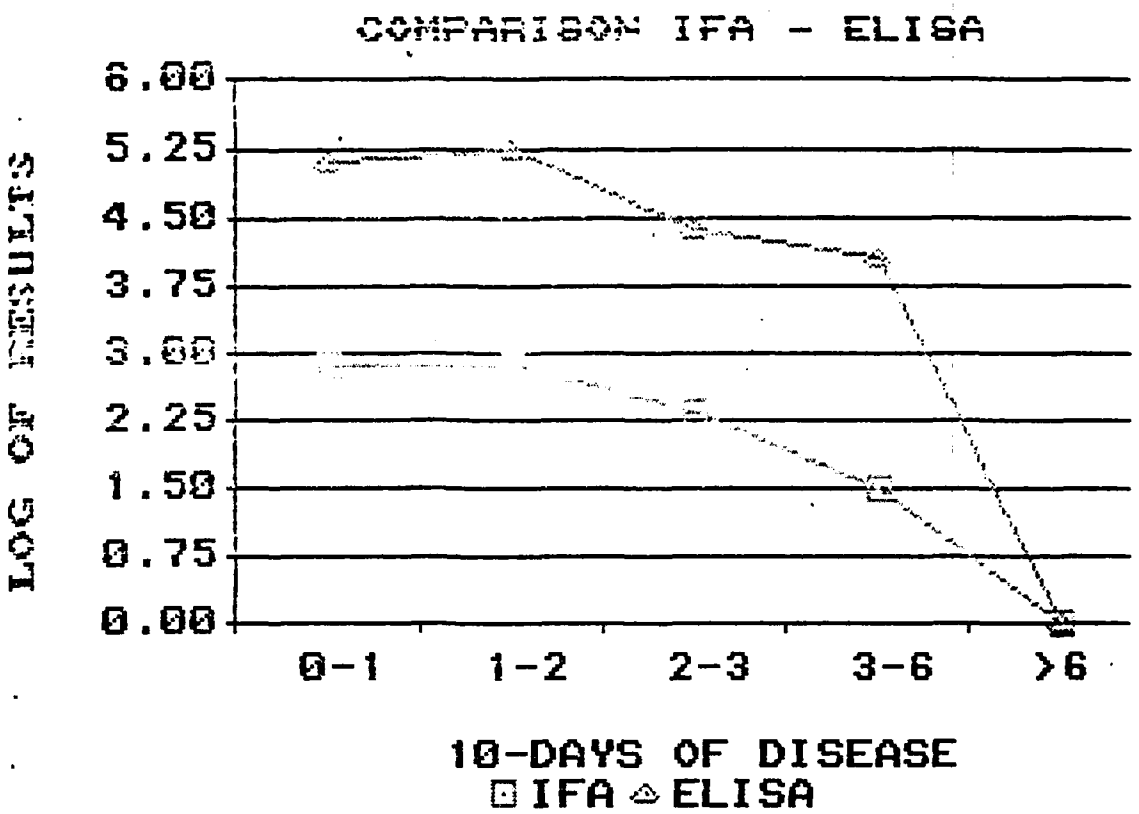

COMPARISON IFA - ELISA
LOG OF RESULTS
6.00
5.25
4.50
3.75
3.00
2.25
1.50
0.75
0.00
0-1
1-2
2-3
3-6
>6
10-DAYS OF DISEASE
IFA
ELISA

Figure 23.

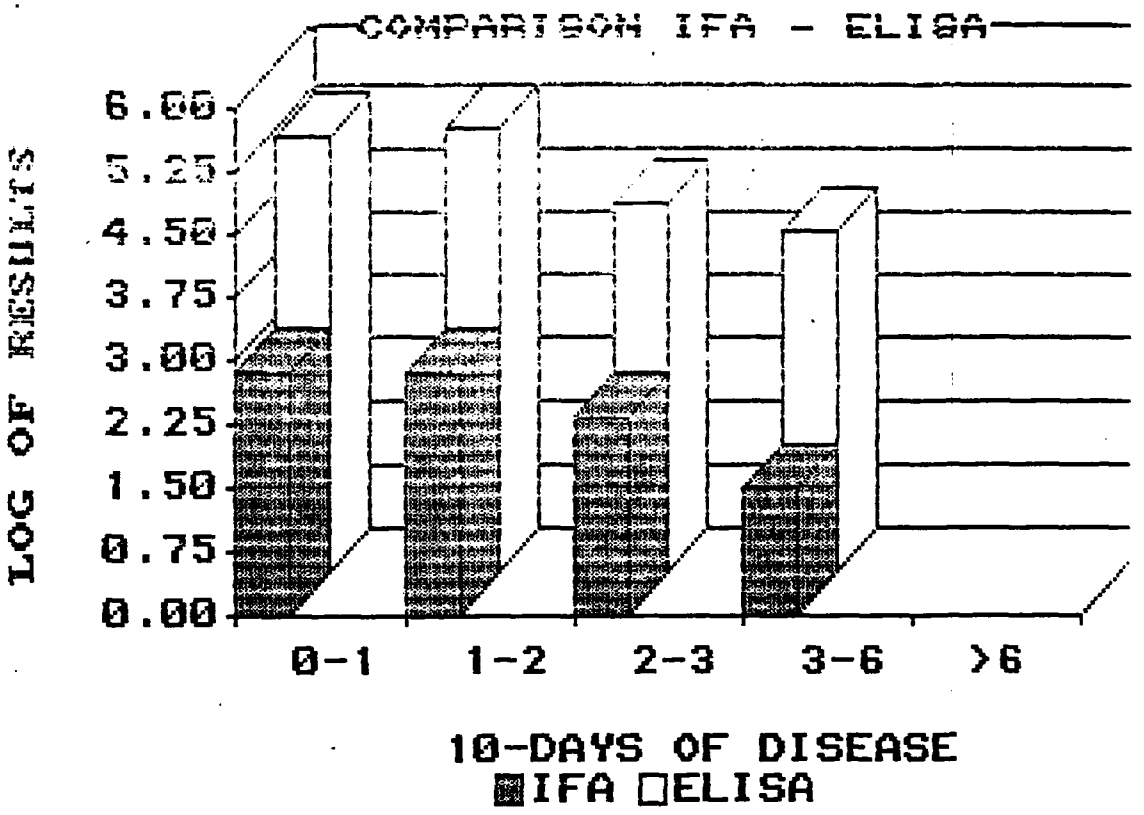

COMPARISON IFA - ELISA
LOG OF RESULTS
6.00
4.50
3.75
3.00
2.25
1.50
0.75
0.00
0-1
1-2
2-3
3-6
>6
10-DAYS OF DISEASE
IFA ELISA

Figure 24.

Fig. 25 : Map of Greece indicating CCHF endemic areas.

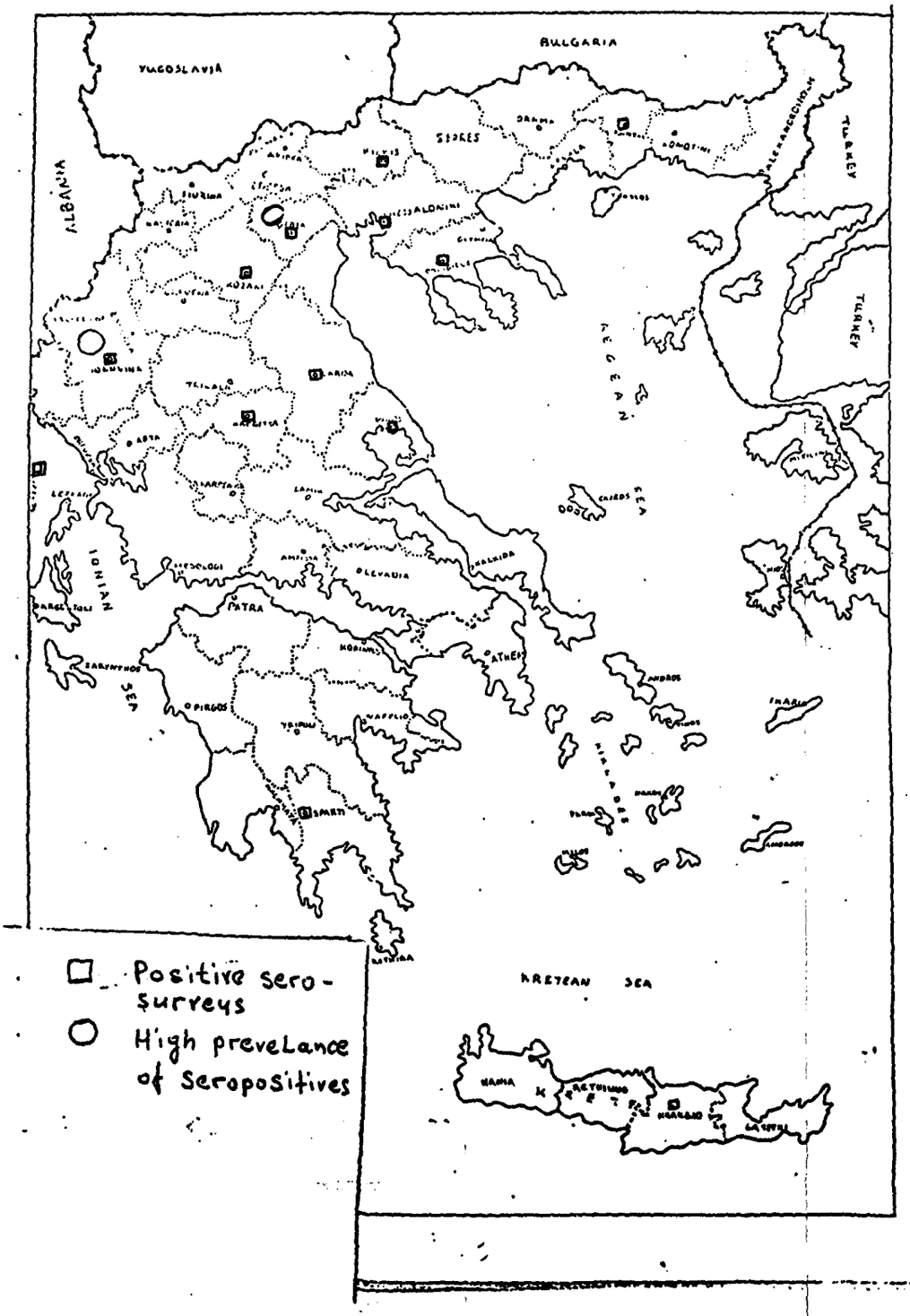


Fig. 26

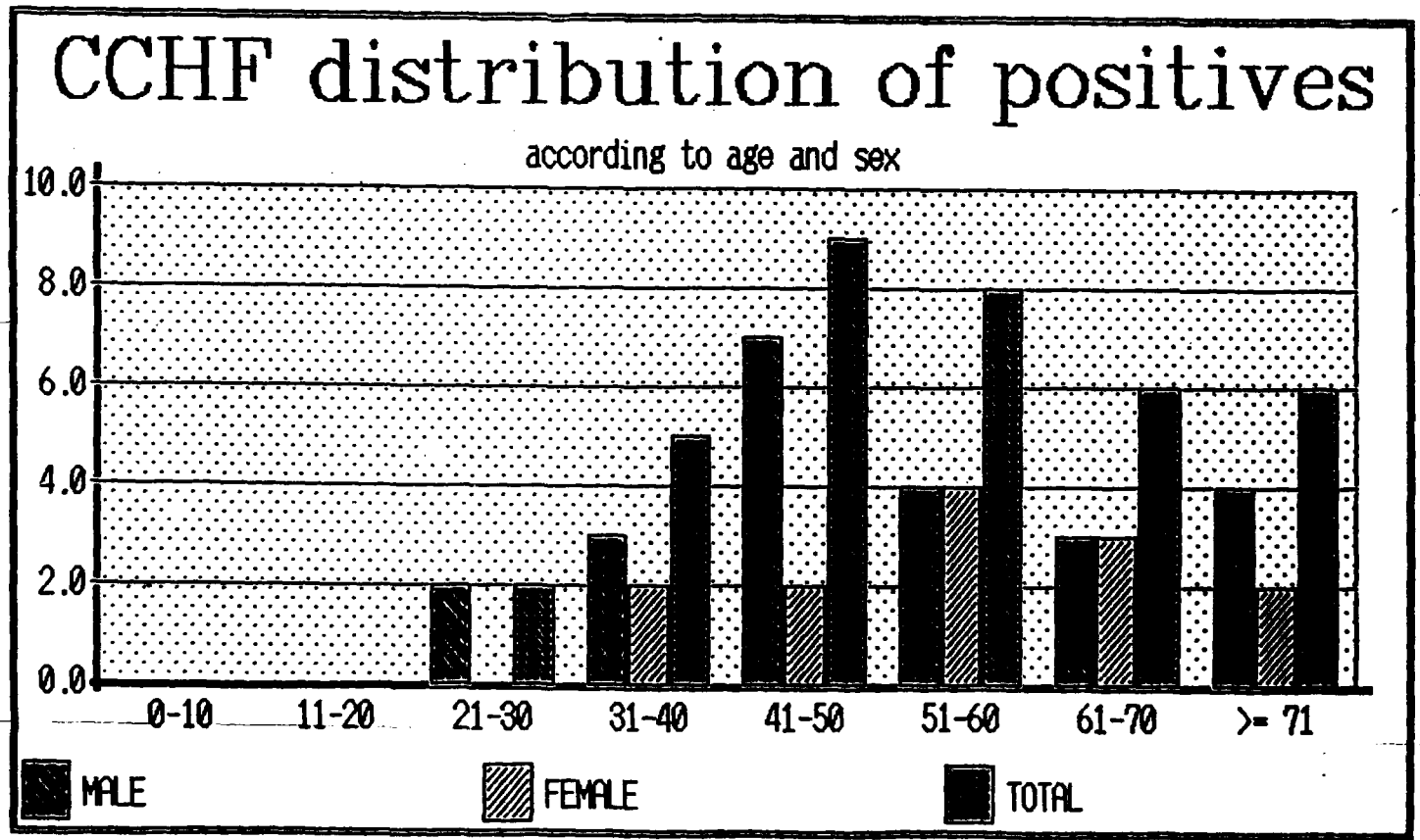

CCHF distribution of positives
according to age and sex
10.0
8.0
6.0
4.0
2.0
0.0
0-10
11-20
21-30
31-40
41-50
51-60
61-70
>= 71
MALE
FEMALE
TOTAL

Fig.27

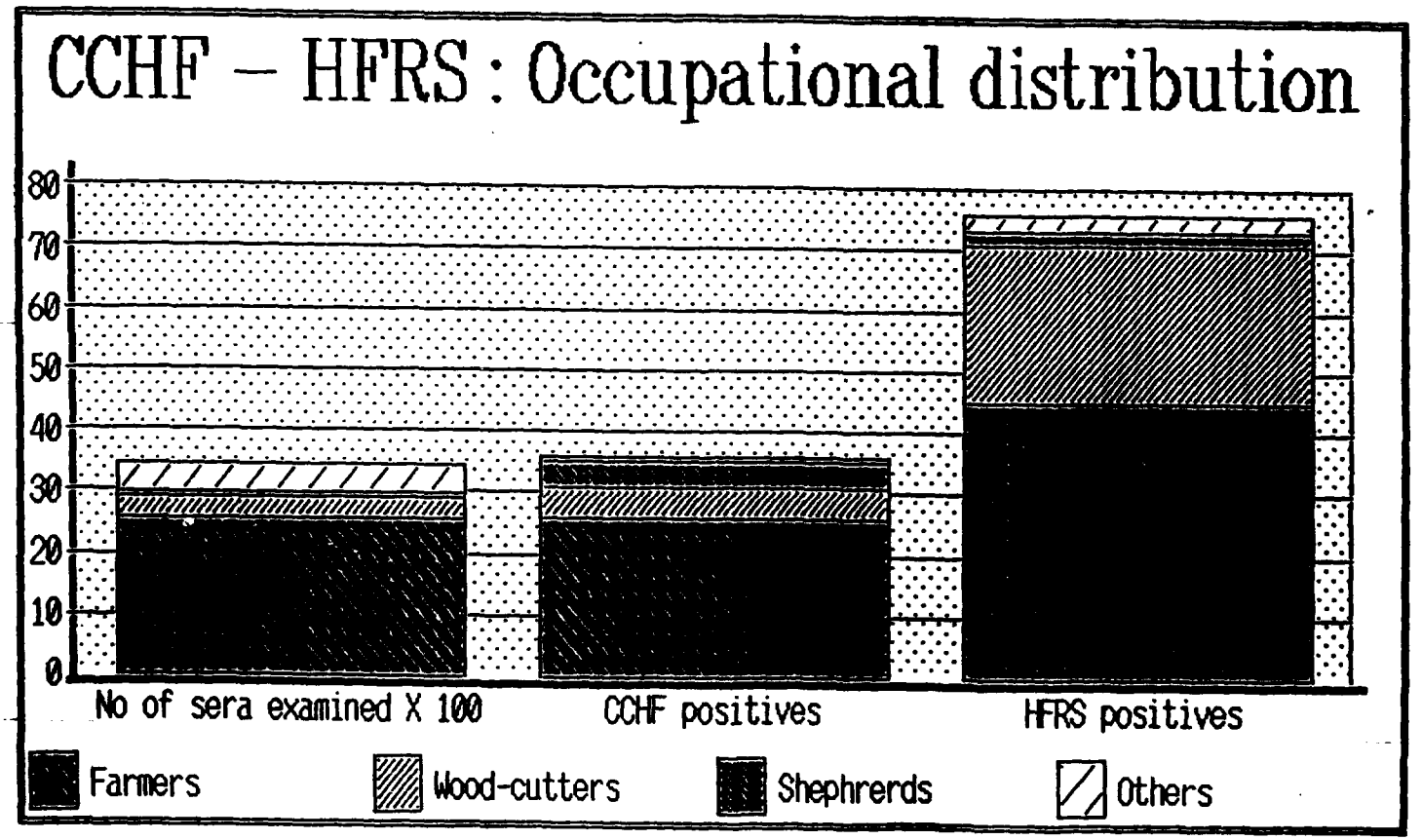

CCHF – HFRS : Occupational distribution
80
70
60
50
40
30
20
10
0
No of sera examined X 100
CCHF positives
HFRS positives
Farmers
Wood-cutters
Shephrerds
Others

Fig. 28: CCHF-Map of Greece indicating the counties where serosurvey was conducted during April 1987 to April 1988.

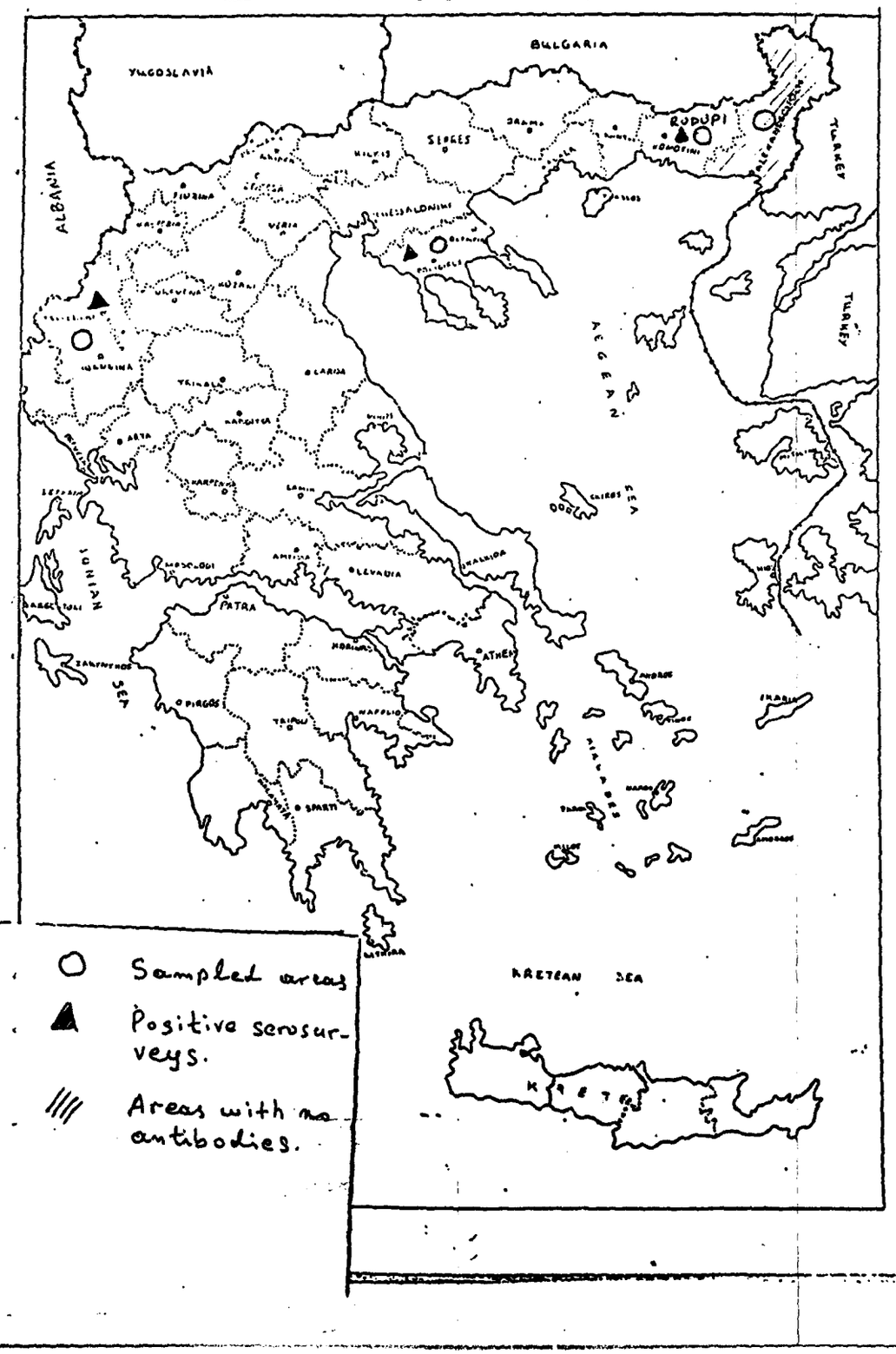


Figure 29 : Map of Bulgaria indicating the intensivity of CCHF virus.

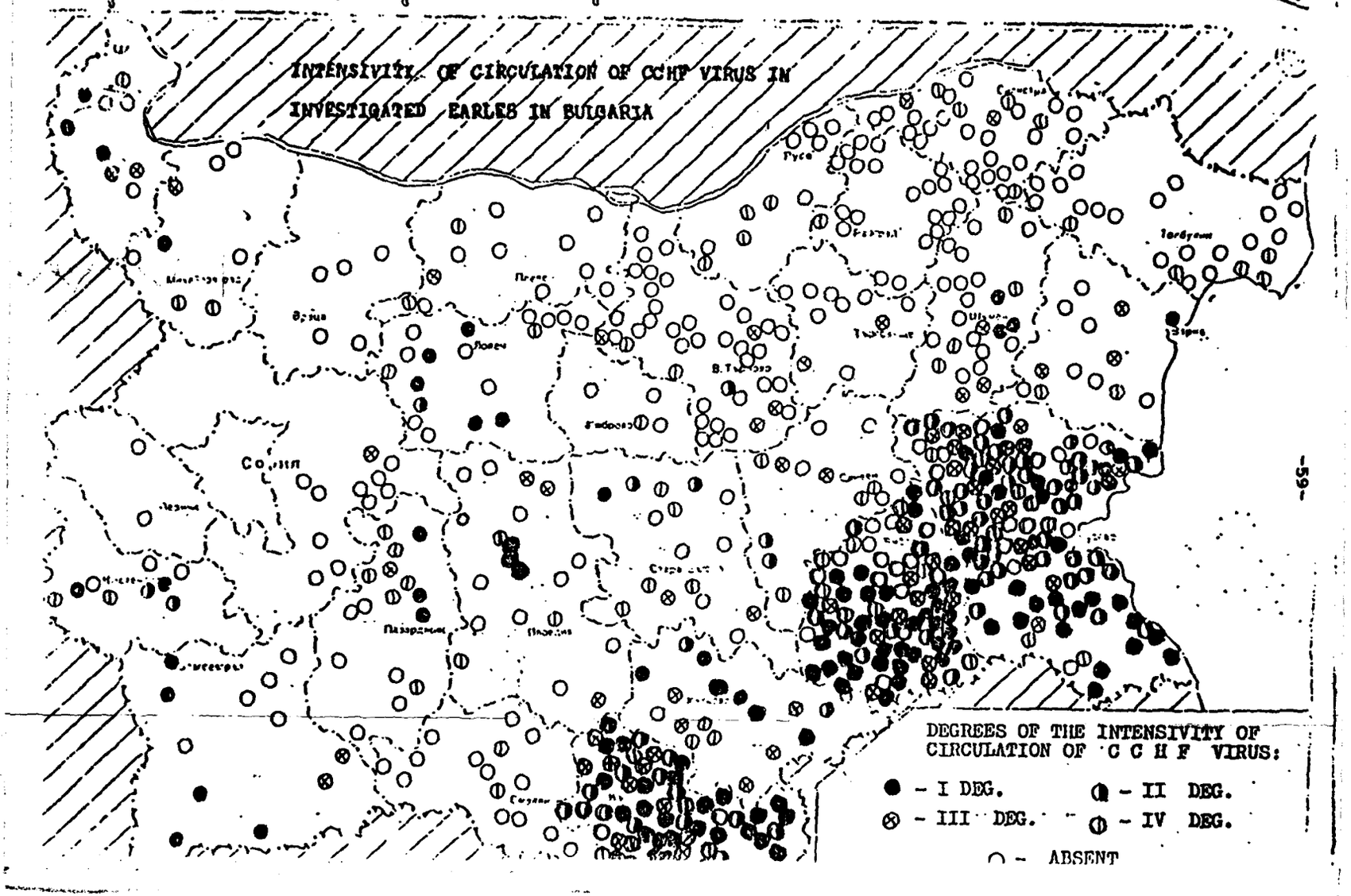
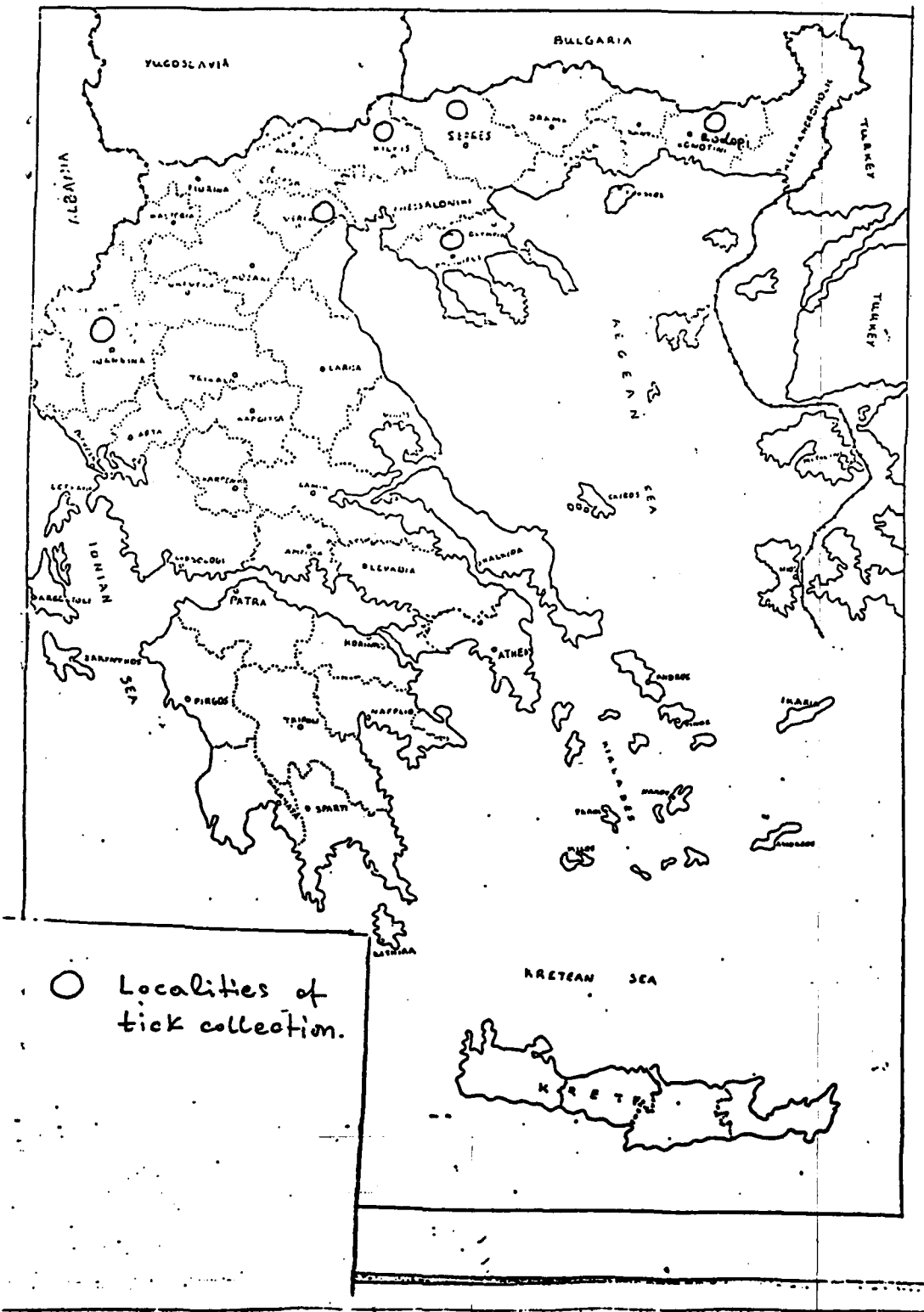


Fig. 30: Map of Greece indicating the localities of tick collection.



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